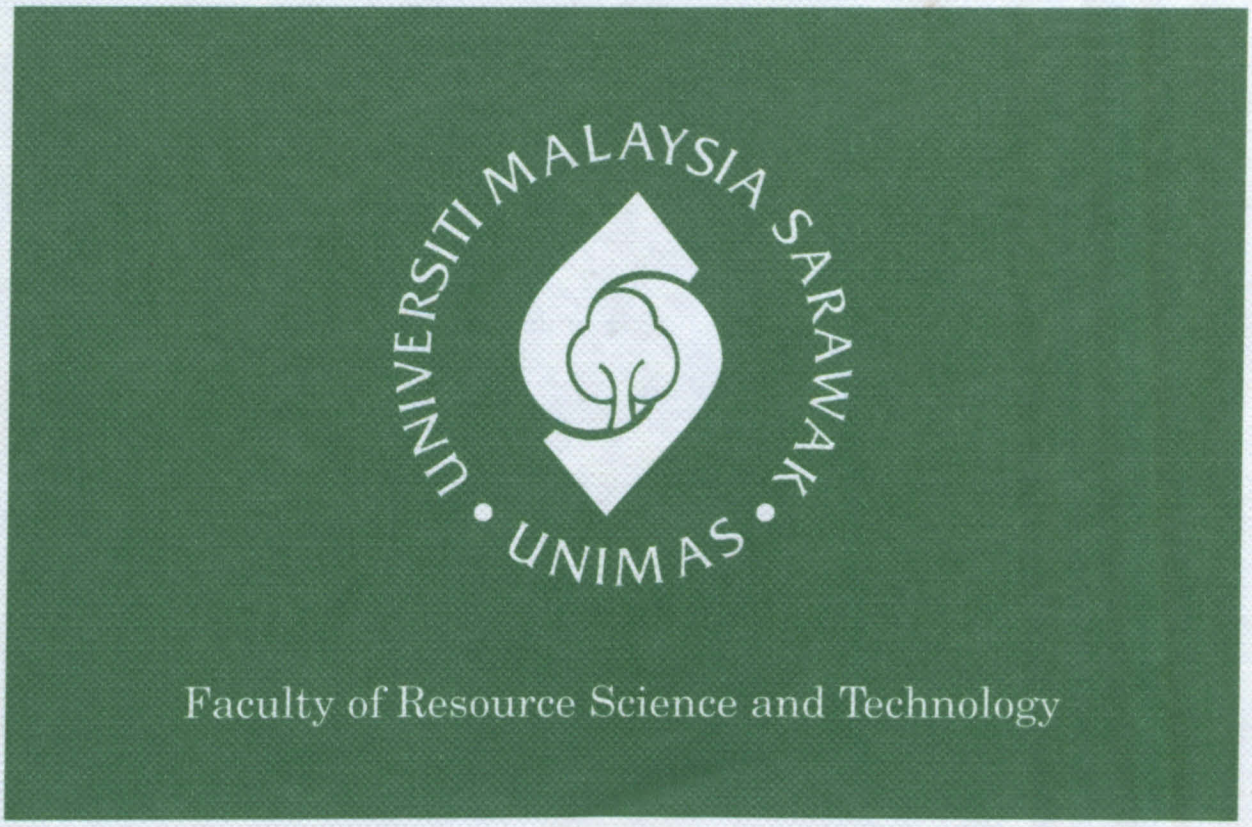


ACCUMULATION OF COPPER, ZINC AND LEAD IN TILAPIA (*OREOCHROMIS* SPP.) UNDER LABORATORY CONDITIONS

Ronny Ak Daud

Bachelor of Science with Honours
(Resource Chemistry)
2007

ACCUMULATION OF COPPER, ZINC AND LEAD IN TILAPIA (*OREOCHROMIS* SPP.) UNDER LABORATORY CONDITIONS

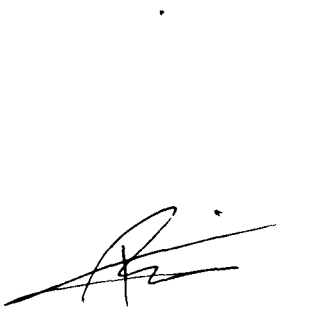
RONNY AK DAUD

This project is submitted in partial fulfillment of
The requirement for the degree of Bachelor of Science with Honours
(Resource Chemistry)

Faculty of Resource Science and Technology
UNIVERSITI MALAYSIA SARAWAK
2007

DECLARATION

No portion of the work referred to in this dissertation has been submitted in support of an application for another degree of qualification of this or any university or institution of higher learning.



Ronny Ak Daud
Program of Resources Chemistry
Faculty of Resources and Technology
Universiti Malaysia Sarawak

ACKNOWLEDGEMENTS

Special thanks to my supervisor, Madam Devagi a/p Kanakaraju, and co-supervisor, Miss Rafeah Wahi for their advise and guidance throughout this research. I also would like to gratefully thank staffs of Department of Chemistry, and lab assistants for their help and support. Special thanks are also due to staffs of Agriculture Research Centre of Semenggok (*Pusat Pengeluaran, penyelidikan dan Latihan Perikanan Darat*) for providing samples for this research to be carried out.

My acknowledgements would not be completed without mentioning my family for their positive encouragement and continuous support, without whom I would barely be who I am.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Accumulation of Copper, Zinc, and Lead in Tilapia (*Oreochomis* spp.) Under Laboratory Conditions

Ronny Ak Daud

Program Kimia Sumber
Fakulti Sains dan Teknologi Sumber
Universiti Malaysia Sarawak

ABSTRACT

The uptake and elimination of copper (Cu), zinc (Zn) and lead (Pb) using tilapia during exposure to these metals was examined under controlled laboratory experiments. Tilapia (*Oreochromis* spp.) was acclimatized at 25± 2° C (Room temperature) for 4 days. The *tilapia* was exposed to sublethal concentrations (LC_{50}) of copper (Cu), zinc (Zn) and lead (Pb). The experiment was designed to allow four days of metal uptake and four days of depuration period. The tilapia was taken out at 24, 48, 72 and 96 hours for metal analysis. The water samples were tested daily and the water temperature of the experimental tanks were kept at 25± 2° C and pH of 7.6 ± 0.1 was maintained. Accumulations of all metals increased with exposure time. Different rates of accumulation and depuration in tissue was found and this could be due to different mechanisms of metal binding and regulation. At the end of depuration, Zn levels in tissues of tilapia (*Oreochromis* spp.) were higher than before exposure, while Cu and Pb levels in tissues were almost similar to levels at 24 hours of exposure. These results indicated that tilapia (*Oreochromis* spp.) can be used as good biomonitoring organism of environment metals contaminant. The positive patterns, although different rates of accumulation and depuration for Cu, Zn and Pb support the use of tilapia (*Oreochromis* spp.) as a biomonitoring agent for such metals.

Key words: Tilapia; Copper; Zinc; Lead; Accumulation; Depuration

ABSTRAK

Kadar penyerapan dan penyingkiran logam kuprum (Cu), zink (Zn) dan plumbum (Pb) oleh ikan tilapia semasa pendedahan terhadap logam-logam berat ini telah dikaji dibawah keadaan yang terkawal dalam makmal. Tilapia (Oreochromis spp.) *diletakkan didalam air yang mempunyai suhu 25± 2° C untuk tempoh 4 hari sebelum didedahkan dengan air yang mengandungi logam berat (Cu, Zn dan Pb). Tilapia telah didedahkan dengan subkepekatan maut (LC_{50}) kuprum, zink dan plumbum. Ujikaji ini telah direkabentuk untuk menbolehkan pendedahan selama 4 hari kepada logam berat dan penyingkiran selama 4 hari. Sampel ikan tilapia telah diambil secara rawak pada masa 24 jam, 48 jam, 72 jam dan 96 jam untuk dianalisis. Sampel air telah diuji setiap hari dimana suhu ditetapkan pada 25± 2° C dan pH ditetapkan pada nilai 7.6 ± 0.1. Penyerapan oleh logam berat meningkat dengan masa pendedahan. Kadar penyerapan dan penyingkiran didapati berbeza antara logam. Ini mungkin disebabkan oleh perbezaan dalam mekanisme tindak balas oleh logam-logam itu. Pada penghujung tempoh penyingkiran logam, kepekatan logam zink dalam tilapia didapati lebih tinggi daripada kepekatan logam pada 24 jam pertama pendedahan logam zink. Keputusan ini menunjukkan bahawa tilapia (Oreochromis* spp.) *merupakan organisma yang dapat digunakan sebagai "biomonitor" yang baik untuk pencemaran logam berat. Tren positif yang ditunjukkan menyokong pengunaan tilapia sebagai" biomonitor" bagi logam berat.*

Kata kunci: Tilapia; Kuprum; Zink; Plumbum; Penyerapan; Penyingkiran

CHAPTER ONE

INTRODUCTION

1.1 Background

Contamination of aquatic ecosystems (lakes, rivers, streams, etc) with metals has been receiving increased worldwide attention. Fish are often at the top of the aquatic food chain and may concentrate large amounts of some metals from the water. Accumulation patterns of contaminants in fish depend both on uptake and elimination rates (Hakanson, 1984).

Heavy metals have been identified as one of the most dangerous pollutants of aquatic ecosystems, due to their persistence and elevated toxicity for many organisms. Transition metals (i.e. copper, zinc, iron, cobalt, selenium, manganese) are essential for the health of most organisms, forming integral components of proteins involved in all aspects of biological function. Their ubiquity is governed by their ability to form a wide range of coordination geometries and redox states, which allows these elements to interact with many cellular entities, performing pivotal roles in cellular respiration, oxygen transport, protein stability, free radical scavenging, and the action of many cellular enzymes, as well as for DNA transcription. However, in excess they are toxic, binding to inappropriate biologically sensitive molecules or forming dangerous free radicals. Consequently, there is a fine balance between metal deficiency and surplus and it is vital for organisms to maintain metal homeostasis *via* balancing absorption and excretion (Nicolas *et al.*, 2003).

All heavy metals are potentially harmful to most organisms at some level of exposure and absorption. The levels of metals in upper members of the food web like fish can reach

values many times higher than those found in aquatic environment or in sediments. Studies carried out with different fish species have revealed that both essential and non-essential metals can produce toxic effects in fish by disturbing physiological activities, biochemical processes, reproduction and growth and mortality (Yilmaz, 2005). Metals are nonbiodegradable, and once they enter the environment, bioconcentration may occur in fish tissue by means of metabolic and biosorption processes. From an environmental point of view, bioconcentration is important because metal ions usually occur in low concentrations in the aquatic environment and subtle physiological effects go unnoticed until gross chronic reactions become apparent. Although trace metals are essential for normal physiological processes, abnormally high concentrations can be toxic to aquatic organisms (Wepener *et al.,* 2001).

Tilapia (*Oreochromis* spp.) is the common name applied to three genera of fish in the family Cichlidae: *Oreochromis, Sarotherodon,* and *Tilapia.* Tilapia *(Oreochromis* spp.*)* is native freshwater fish of Africa (Trewawas, 1983). The expression is derived from the African native Bechuana word "thiape," meaning fish. Cichlids are well known as colorful aquarium fish, and for their ability to adapt to new environments. Cichlids also display highly organized breeding activities. Tilapia are omnivores which have a diversified food spectrum (Huet, 1994). According to Wong *et al* (1996), tilapia can survive by taking food such as crustaceans, debris, vascular plants, and microalgae. They can survive at adverse environmental conditions because their resistance to disease is strong; their respiratory demands are slight so they can tolerate low oxygen and high ammonia levels. Even some fresh water tilapia are able to survive and grow over a wide range of salinities (Watanabe *et al.*, 1987). It has been noted that heavy metals were accumulated in tilapia after they were fed with metal contaminated sludge (Wong and Chiu, 1993).

Ecotoxicology or toxicology is a scientific discipline based on the study of modifications in ecosystems which undergo long or short-term disruptions. Ecotoxicology studies the chemical affects on the organisms and to understand the concentration of chemicals at which organisms are affected. There are two main needs for measuring the ecological effects of chemicals, which are to anticipate how toxicants can impact ecological system and to assess the changes that take place in the systems under the influence of released toxic substances. The terms transfer and accumulation are closely linked, the first representing a change of state for the second. Bioaccumulation occupies an important place it is the result of the processes by which the contaminant enters an organism and the process of decontaminating, a combination of the mechanisms of excretion into the surrounding environment, and endogenous biotransformation. A detailed analysis provides a very useful tool for understanding the mechanisms of accumulation (Boudou and Ribeyre, 1989).

This study was undertaken to quantify the accumulation of heavy metals (copper, zinc and lead) within the tissues of tilapia. This fish is considered as one of the commercial fish for both the fisheries and the local inhabitants as a potential source of food. Studying the relation between the biological parameters of fish and the tendency of metal accumulation will provide some information about the environmental state. This will be useful as an alarm signal to minimize the rate of pollution of heavy metals in the lake and for the management programs of the lakes.

1.2 Statement of Problem

Investigations of metals accumulation and assessment of fish as biomonitor under field and experimental conditions have been done on various species of fish such as Catfish (*Clarias*

gariepinus), Grey Mullet (*Mugil cephalus L.*), Sea Bream *(Sparus aurata L.*) and other species of fishes. Various species of tilapia also have been used to perform toxicity test such as Nile tilapia (*Oreochromis niloticus*), *Tilapia mossambica* and *Tilapia zillii.* Data on metals accumulation in tilapia in local environment, specifically Sarawak is scarce. Hence, this study was undertaken to address the capability of tilapia to be used as a biomonitor in Sarawak.

1.3 Objectives

The objectives of this study were to determine the lethal concentration of copper (Cu), zinc (Zn) and lead (Pb) in tilapia (*Oreochromis* spp.), to quantify the accumulation and maximum exposure of heavy metals (Cu, Zn and Pb) in tissue of tilapia (*Oreochromis* spp.) and to perform statistical analysis in order to determine if there is any significant difference in the concentrations of the heavy metals in the accumulation and elimination patterns of copper (Cu), zinc (Zn) and lead (Pb) in tilapia (*Oreochromis* spp.).

CHAPTER TWO

LITERATURE REVIEW

2.1 Heavy Metals

The presence heavy metals in different foods constitute serious health hazards, depending on their relative levels. For example, cadmium and mercury injure the kidney and cause symptoms of chronic toxicity, including impaired kidney function, poor reproductive capacity, hypertension, tumors and hepatic dysfunction. Lead causes renal failure and liver damage. Some other metals (e.g. chromium, zinc and copper) cause nephritis, anuria and extensive lesions in the kidney (Mansour and Sidky, 2002)

Heavy metal is often used as a group name for metals and semimetals (metalloids) that have been associated with contamination and potential toxicity or ecotoxicity (John, 2002). "Heavy metals" is an inexact term used to describe more than a dozen elements that are metals or metalloids (elements that have both metal and nonmetal characteristics). Examples of heavy metals include chromium, arsenic, cadmium, lead, mercury, and manganese. Generally, heavy metals have densities above 5 g/cm^3. Because they cannot be degraded or destroyed, heavy metals are persistent in all parts of the environment. Human activities affect the natural geological and biological redistribution of heavy metals through pollution of the air, water, and soil. The primary anthropogenic sources of heavy metals are point sources such as mines, foundries, smelters, and coal-burning power plants, as well as diffuse sources such as combustion by-products and vehicle emissions (Hawkes, 1997).

Heavy metals are natural trace components of the aquatic environment, but their levels have increased due to industrial, agricultural and mining activities. As a result, aquatic animals are exposed to elevated levels of heavy metals (Ünlü and Gümgüm, 1993). Some heavy metals such as zinc, copper and cobalt are essential in trace amounts for normal growth and development; however, others such as mercury, cadmium and lead have no biological importance (Canl and Furness, 1993). All heavy metals are potentially harmful to most organisms at some level of exposure and absorption (Larsson *et al.*, 1985). The levels of metals in upper members of the food web like fish can reach values many times higher than those found in aquatic environment or in sediments (Ünlü and Gümgüm, 1993).

2.1.1 Copper

Copper is a common pollutant in surface waters and its toxicity is largely attributable to its cupric (Cu^{2+}) form, which is the species commonly found or readily complexed by inorganic and organic substances and adsorbed onto particulate matter. Complexed copper is biologically unavailable but plants and animals may absorb some copper in the environment. In the unpolluted water, copper may be less than 5 μg/L (Alabaster and Lloyd, 1982). Copper compounds are used for prophylactic purposes to control fish diseases and parasites (Moore *et al.*, 1984). Copper compounds are also used to control algae, kill slugs and snails in irrigation water systems and municipal water treatment systems.

Copper acts as a co-factor for a number of key proteins (i.e. superoxide dismutase, ceruloplasmin). Copper's flexible redox state means it plays a vital role in cellular respiration, with cytochrome *c* oxidase being an important copper protein. Copper is thus an essential element, and daily dietary requirements for fish are in the region of 15–60 μmol (1–4 mg) Cu

kg^{-1} dry mass (Lanno *et al.*, 1985; Watanabe *et al.*, 1997). However, in excess, copper is toxic. From a dietary perspective, the primary toxic action is predominantly the production of free radicals in tissues where copper accumulates. In addition, dietary copper toxicity can occur at several other loci in the gut and includes inhibition of digestive enzymes and reduced gut motility (Woodward *et al.*, 1995).

Conversely, elevated level of copper may become acutely or chronically toxic to aquatic lives. While acute effects may be death, chronic effects could be reduced growth, shorter lifespan, reproductive problems, reduced fertility and behavioural changes (Laurén and McDonald, 1985).

The toxicity of copper to aquatic life varies with the physical and chemical conditions of the water. Factors like water hardness, alkalinity, pH, dissolved oxygen (DO) and temperature affect the toxicity of copper. The toxicity of copper has also been found to reduce in the presence of organic or inorganic substances like suspended solids since complexes adsorption occur with these substances (Oronsaye and Ogunbor, 1998). At low concentrations, copper is an essential element for both plants and animals since it is an important component of enzymes and carries oxygen in crustaceans such as shrimps and lobsters (Copperinfo, 2001).

2.1.2 Zinc

Zinc is essential due to its vital structural and catalytic importance in more than 300 proteins that play important roles in piscine growth, reproduction, development, vision and immune function (Watanabe *et al.*, 1997). Consequently for fish, of the essential metals, zinc is second in quantitative importance only to iron (Watanabe *et al.*, 1997). Dietary zinc requirements ranged

between 230–460 μmol (15–30 mg) kg^{-1} dry mass of diet (Ogino and Yang, 1978; Gatlin and Wilson, 1983).

Zinc is governed by its ability to form a wide range of coordination geometries, allowing it to interact with a wide range of cellular entities (Vallee and Falchuk, 1993; McCall *et al.*, 2000). Furthermore, zinc is redox inert, enabling the formation of relatively stable associations within the cellular environment (Vallee and Falchuk, 1993). Consequently, in contrast to copper and iron, zinc does not form free radical ions, and in fact has antioxidant properties (Powell, 2000). Zinc generates toxicity to fish by interfering with calcium homeostasis (Spry and Wood, 1985; Hogstrand and Wood, 1996).

2.1.3 Lead

Lead has a combination of physical and chemical properties that make it extremely useful industrially. Major use of lead is in battery production since a large drop has occurred in the demand for gasoline additives containing lead. In the past, lead was used in the chemical industry for preparing paints, pigments, and colored inks were widespread, but many countries have now restricted this use. The natural concentration of lead in surface water has been estimated 0.02 $\mu g.L^{-1}$ and it rarely exceeds a few micrograms. L^{-1}. However, high levels of lead are associated with areas in the vicinity of lead mines, smelteries and battery-producing industries (WHO, 1995).

Sub-lethal toxicity of lead to fish produces hematological and neurological effects (Hodson *et al.*, 1980). It is well known that lead causes early mortality of mature red blood cells

and inhibition of hemoglobin formation through inhibition of erythrocyte δ-amino levulinic acid dehydratase (ALA-D). The result is anemia at high lead exposures or compensating erythropoiesis at lower exposures (Hodson, 1976). Neurological effects include impaired learning behaviour, darkening of the caudal region (black tails), and eventual spinal curvatures (Hodson *et al.*, 1978, 1979, 1980).

2.2 Tilapia (*Oreochromis* spp.)

Tilapia consists of three aquaculturally important genera *Oreochromis, Sarotherodon* and *Tilapia*. All tilapia species are nest builders; fertilized eggs are guarded in the nest by a brood parent. Tilapia are shaped much like sunfish or crappie but can be easily identified by an interrupted lateral line characteristic of the Cichlid family of fishes. They are laterally compressed and deep-bodied with long dorsal fins. The forward portion of the dorsal fin is heavily spined. Tilapia ingests a wide variety of natural food organisms, including plankton, some aquatic macrophytes, planktonic/ benthic aquatic invertebrates, larval fish and decomposing organic matter. Tilapia are often considered filter feeders because they can efficiently harvest plankton from the water. The gills of tilapia secrete a mucous that traps plankton. Tilapia survives routine dawn dissolved oxygen (DO) concentrations of less than 0.3 mg/L. Tilapia can survive in pH ranging from 5 to 10 but do best in a pH range of 6 to 9 (Thomas and Michael, 1999).

According to Mair and Roberts, (1988), tilapia are easily growing fish species since they eat variety of foods, resist to diseases and grow well in poor quality water with low dissolved oxygen Some tilapia can either survive in fresh, brackish or sea water. Tilapia are more tolerant than most commonly farmed freshwater fish to high salinity, high water temperature, low

dissolved oxygen, and high ammonia concentrations. Reproductive performance of tilapia begins to decline at salinities above 10 to 15ppt. The lower lethal temperature for most species is 50 to 52°F for a few days. Tilapia generally stops feeding when water temperature falls below 63°F. Reproduction is best at water temperatures higher than 80°F and does not occur below 68°F. Optimal water temperature for tilapia growth is about 85 to 88°F (Thomas and Michael, 1999).

Tilapia exhibit maximum growth rates at temperatures between 25 and 30°C, making them more likely to become established and invasive in tropical climates. However, both tolerances to water temperature and to salinity vary greatly between species. Tilapia are well adapted to artificial culture environments, gain weight quickly at optimum conditions and reproduce on the farm without special management or infrastructure (Wong *et al.*, 1996).

2.3 Toxicity Tests

The impact of metals, as well as other pollutants, aquatic biota can be evaluated by toxicity which is used to detect and evaluate the potential toxicological effects of chemicals on aquatic organisms. However, little research has been done on impact of contaminants on tropical ecosystems (Lacher and Goldstein, 1997). At present, toxicological guidelines for metals in most tropical countries generally derived from data collected in nontropical ecosystems (Oliveira *et al.*, 1996).

There are two ultimate aims in ecotoxicology study which are to predict and to diagnose the causes of ecological and biological effects which result from exposure to heavy metals and environmental stress (Yap *et al.*, 2003). Most toxicity test have been concerned with measure of

acute lethality and the result are expressed as a concentration or dose of toxicant at which a specified percentage (example LC_{50}) of the test organisms are killed over a standard period of time (examples 24, 48, 72, or 96 hours) (Guven *et al.*, 1999).

2.3.1 Acute Toxicity Test (LC_{50})

The LC_{50} is an estimate of the percentage of effluent that will cause 50% mortality in the test species. It is calculated using a probit regression with 95% confidence limits. This analysis consists of transforming the observed proportion of mortalities with a probit transformation, and transforming the treatment concentrations to $\log_{10}$. The relationship between the above and from this a regression is used to determine the LC_{50} and 95% confidence limits (Hall and Golding, 1998).

During the acute toxicity test, the fish are normally exposed to a series of toxicant concentrations, which on the basis of simple range finding tests, are expected to give responses from 0% to 100%. Acute tests are those in which exposure to the toxicant results in significant effects or responses detectable in a short period of time, normally within 24 hours till 96 hours. The concentration at which the median response occurs in a predetermined time can be calculated using the median lethal concentration (LC_{50}) (Boudou and Ribeyre, 1989). LC_{50} can be defined as median lethal concentration of a chemical in air but in environmental studies it can also mean the concentration of a chemical in water.

2.4 Toxicity Test Using Fish

Among aquatic organism, fish are valuable biomonitor of bioaccumulation of toxic compounds. *Tilapia mossambica*, a freshwater fish, has been used to study the toxicity and accumulation of tributyltin as well as heavy metals from sewage- fed ecosystems. The direct accumulation of arsenic by *tilapia mossambica* was proportional to the concentrations of arsenicals in water, and small amounts of accumulated arsenic were partially transformed to methylated arsenic up to TMA (trimethylarsenic) species (Suhendrayatna *et al.*, 2001).

Study of chronic dietary copper toxicity in Nile tilapia revealed that fish accumulated excess Cu in the liver and intestine, and showed a decline in growth and nutritional performance which is associated with liver pathology. Importantly, Nile tilapia does not recover quickly from dietary Cu exposure. Compensatory growth did not occur and the liver showed further increases in Cu content and fatty change during the recovery phase (Richard and Benjamin, 2005).

According to Mazon and Fernandes (1999), juvenile specimens of *P. scrofa* are potential vertebrate bioindicator organisms for environmental monitoring. In order to evaluate lead effects, they examined gill morphology, hematocrit, blood sodium, glucose, lipids, protein, and cholesterol of *P. lineatus* exposed to two sublethal lead concentrations. Lead can affect glucose metabolism as reported by Salmerón-Flores *et al* (1990), who reported increased glucose blood concentration in *Sarotherodum aureus* in response to lead exposure.

Lethal and sub-lethal effects of copper on African catfish *(Clarias gariepinus)* juveniles were studied using a 96-hour static bioassay. Five standard concentrations 0.0, 1.8, 3.2, 5.6, and 10.0 mg/L were prepared (coded A–E respectively). The 96-hour LC_{50} estimated using the

logarithm methods were 0.6, 0.71 and 0.7 mg/l with mean as 0.67 mg/l. The mean copper concentration in bone ranged from 1.86 to 17.04 ppm and muscle from 1.29 to 55.5 ppm. There were significant differences ($P<0.05$) in mortality among treatments (Olaifa *et al.*, 2004).

The studies carried out with different fish species have revealed that both essential and non-essential metals can produce toxic effects in fish by disturbing physiological activities, biochemical processes, reproduction and growth and mortality (Larsson *et al.*, 1985; Weis and Weis, 1989; Abel and Papoutsouglou, 1986). Vera *et al* (1998) reported that the liming agent ($CaCO_3$) tends to protect tilapia (*Oreochromis mossambicus)* against copper hepatoxicity.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Sample collection and preparation

Tilapia fish (size: 3-4 cm, weight: 10-15 g) were obtained from Agriculture Research Centre, Semenggok, Kuching (*Pusat Pengeluaran, penyelidikan dan Latihan Perikanan Darat*). Fish samples were brought to the laboratory and they were allowed to acclimatise in the holding tanks for at least four days prior to metal exposure. At the laboratory, the samples wet weight were determined.

3.2 Acclimatization

Tilapia fish (size: 3-4 cm, n= 175 fish) were placed into five experimental tanks (n=35 fish per tank) with dechlorinated tap water at 25± 2° C (Room temperature) and aeration pump system. The dechlorinated tab water was changed from time to time during the acclimatization test. The fish were fed daily with a control diet (pellet fish food with no added heavy metal) for four days in order to acclimate to experiment conditions.

3.3 Accumulation and Elimination Test

The accumulation study was consisting of three single elements. The tilapia was exposed to sublethal concentrations (LC_{50}) of copper (Cu), zinc (Zn) and lead (Pb). The experiment was designed to allow four days of metal uptake and four days of elimination period in metal free water tank with clean water. The *tilapia* was taken out at 24, 48, 72 and 96 hours for metal

analysis. The water temperature of the experimental tanks was kept 25± 2° C (room temperature) and a pH of 7.6 ± 0.1 was maintained. All experiments were conducted using experimental tanks. Metals concentration was prepared using dechlorinated tap water as diluents from stock solutions (1000 mg/L) of copper sulfate ($CuSO_4.5\ H_2O$), zinc sulfate ($ZnSO_4.7\ H_2O$), and $Pb(NO_3)_2$. Test containers or experimental tanks with metal solutions were allowed to equilibrate for 48 hours before the experiment started to minimize adsorption and consequent loss of the metals (Suhaimi- Othman and Pascoe, 2007). Table 3.1 showed the concentration of copper, zinc, and lead used in the toxicity test.

Table 3.1: Concentration of metals used in the toxicity test

	Cu	Zn	Pb
Tank 1	0.0 ppm	0.0 ppm	0.0 ppm
Tank 2	5.0 ppm	10.0 ppm	1.0 ppm
Tank 3	10.0 ppm	15.0 ppm	2.0 ppm
Tank 4	15.0 ppm	20.0 ppm	3.0 ppm
Tank 5	20.0 ppm	25.0 ppm	4.0 ppm

None of the fish were fed during the accumulation and depuration period in order to empty the gut and to facilitate dissection. Every 24 hours, four tilapia were randomly sampled from each tank for tissue analysis.

3.4 Chemical Analysis

Water samples were taken after 96 hours (at the end of the accumulation test). Water samples were also taken at the end of the depuration test. Water samples were filtered using 0.45 μm membrane filter and acidified with nitric acid (70%). After acidification the water samples, total concentrations of Cu, Zn and Pb were measured by Flame Atomic Absorption Spectrophotometer (FAAS). Other parameters such as pH and temperature of the water were monitored.

Tilapia samples were taken out every 24 hours throughout the uptake and depuration phases for metals analysis. For metal analysis, the samples were freeze-dried and whole tissue of each fish were dissected, weighed and dried at 105 °C until they reached to a constant weight. The samples were thawed. 0.5 g of tissues were weighed and placed in beakers and 10 ml of freshly prepared nitric acid and hydrogen peroxide (1:1) v/v according to Daziel and Baker, (1983) methods were added and set aside till the initial reactions subsided. Each beaker on a hot plate and gently boiled at a temperature not exceeding 160^0C for about 2 hours to reduce the volume to 3-4 ml. Then, it was cooled and transferred to a 25 ml volumetric flask and made up to volume with de-ionised water (FAO/ SIDA, 1993). Then, samples were tested for heavy metal (Cu, Zn, and Pb) using Flame Atomic Absorption Spectrophotometer (FAAS).

3.5 Metal Analysis

After filtration, samples were analysed for copper (Cu), zinc (Zn) and lead (Pb) in an air-acetylene FAAS (Perkin- Elmer Model 3110). To avoid possible contamination, all glassware and equipment that are being used were acid- washed and the accuracy of the analysis was

checked against blanks and with standard addition procedure. Water sample were also analysed. Samples were filtered and analysed by using the FAAS (Perkin- Elmer Model 3110).

3.6 Data Analysis

Stock solutions of the heavy metals (Cu, Zn and Pb) were freshly prepared and were added to few test vessels containing deionized water to achieve the required range of metals concentrations. Initial range- finding experiments were performed to derive the concentrations suitable for LC_{50} determinations. Concentrations showed in Table 3.1 were determined based on the recommended concentration by Handy and Shaw (2006) and Wang and Zhang (2006). Following the metal exposure, samples were checked daily for mortality (Guven *et al.*, 1999).

Calculation for LC_{50}:

$$LC_{50} = \frac{\text{Concentration when 50\% of the samples died}}{\text{The duration of time}}$$

3.7 Statistical Analysis

Two-Factor Without Replication (ANOVA) was used to test whether the data means of the groups are different. Thus, Two-Factor Without Replication (ANOVA) was applied in this study to test the significant differences in the concentrations of the heavy metals in the accumulation and elimination patterns of copper (Cu), zinc (Zn) and lead (Pb) in tissue of tilapia *(Oreochromis* spp.*)*.

3.8 Instrumentation and Calibration

3.8.1 Instrumentation

The samples were analyzed using Atomic Spectroscopy Perkin Elmer Model 3110.

Table 3.2 showed the operating parameters used in AAS operation.

Table 3.2: Parameters used in AAS operation

Elements	Wavelength (nm)	Detection limit (mg/L)
Copper (Cu)	324.8	0.001
Zinc (Zn)	213.9	0.008
Lead (Pb)	283.9	0.01

3.8.2 Calibration

The elemental concentrations were obtained from calibration curve. Table 3.3 showed the range of calibration standards used for metal analysis. The standards used were different for each element.

Table 3.3 Calibration data used for metal analysis

Elements	Calibration standard (ppm)	R^2 value of calibration curve
Copper (Cu)	0.0- 5.0	0.99953
Zinc (Zn)	0.0- 5.0	0.98251
Lead (Pb)	0.0- 5.0	0.99446

* 0.0ppm indicating blank

3.9 Data Treatment

Concentration of solution = A mg/L (from AAS)

Solution = 25mL

Amount of metal weight = Concentration x Volume

= A mg/L x (25mL / 1000mL/L)

= 0.025 A mg

Amount of metal in sample = 0.025 A mg

Concentration of metal in sample = 0.025 A / sample weight

= 0.025 A mg / (0.5g/ 1000 g/kg)

= 50 A mg kg^{-1}

CHAPTER FOUR

RESULT AND DISCUSSIONS

4.1 Metal Concentration in Water

Mean pH and temperature of water samples during the test were 7.6±0.1 and 26±2°C respectively. Concentration of metals in water were determined at 96 hours (at the end of accumulation) and at 192 hours (at the end of depuration). Table 4.1a – 4.1c shows the concentration of copper, zinc and lead in water at 96 hours (at the end of accumulation) and at 192 hours (at the end of depuration). All control samples at 96 hours showed the lowest metal concentrations. However, some control samples sampled at 192 hours slightly exceeded the concentration found in the test tanks. From Table 4.1a - 4.1c, it can be observed that the concentration of heavy metals (Cu, Zn and Pb) in water at the end of the accumulation test were higher than the concentration of metals in water at the end of the depuration test except for control water. The concentration of heavy metals (Cu, Zn and Pb) in control water were almost similar to the concentration of heavy metals (Cu, Zn and Pb) in the water at the end of the depuration test because both water sample were not injected with copper, zinc and lead.

Table 4.1a: Copper concentration in water

Water Sample	N	Concentration of Copper (mg/l)			
		96 hours		192 hours	
		First run	Second run	First run	Second run
Control	3	0.07 ± 1.32	0.08 ± 0.77	0.07 ± 0.86	0.047 ± 0.87
5 ppm	3	3.14 ± 0.50	3.21 ± 0.50	0.05 ± 0.68	0.057 ± 0.16
10 ppm	3	5.71 ± 0.29	5.80 ± 0.29	0.06 ± 0.39	0.063 ± 1.29
15 ppm	3	7.89 ± 0.58	8.13 ± 0.58	0.08 ± 1.68	0.053 ± 0.29
20 ppm	3	11.56 ± 0.75	11.84 ± 1.25	0.08 ± 0.87	0.070 ± 0.58

Table 4.1b: Zinc concentration in water

Water Sample	N	Concentration of Zinc (mg/l)			
		96 hours		192 hours	
		First run	Second run	First run	Second run
Control	3	0.41 ± 0.56	0.39 ± 1.04	0.37 ± 1.00	0.38 ± 0.29
10 ppm	3	6.11 ± 0.50	6.23 ± 0.88	0.38 ± 0.76	0.36 ± 0.58
15 ppm	3	9.17 ± 0.87	8.77 ± 0.29	0.38 ± 1.42	0.39 ± 0.00
20 ppm	3	13.75 ± 0.29	13.47 ± 3.55	0.38 ± 1.53	0.28 ± 1.04
25 ppm	3	17.72 ± 0.76	17.39 ± 0.58	0.39 ± 3.45	0.36 ± 0.46

Table 4.1c: Lead concentration in water

Water Sample	N	Concentration of Lead (mg/l)			
		96 hours		192 hours	
		First run	Second run	First run	Second run
Control	3	0.07 ± 0.50	0.06 ± 1.00	0.04 ± 3.46	0.04 ± 0.29
1 ppm	3	0.75 ± 0.18	0.87 ± 0.49	0.04 ± 0.58	0.06 ± 0.87
2 ppm	3	1.19 ± 2.45	1.20 ± 0.50	0.04 ± 0.40	0.05 ± 0.57
3 ppm	3	1.41 ± 0.29	1.47 ± 0.76	0.07 ± 0.63	0.05 ± 0.00
4 ppm	3	2.57 ± 0.29	2.42 ± 1.33	0.07 ± 0.50	0.04 ± 0.50

4.2 Copper Accumulation and Depuration Test

The mean measured copper concentration (with standard deviation, SE) for 0.0 ppm, 5.0 ppm, 10.0 ppm, 15.0 ppm and 20.0 ppm Cu exposures were 5.92 mg/kg (0.68), 63.44 mg/kg (2.99), 67.92 mg/kg (2.38), 81.10 mg/kg (2.40), and 92.73 mg/kg (2.52), respectively. Figure 4.2a shows that the concentrations of copper in tissues of tilapia increased with increasing exposure time. Statistical analyses indicated significant differences ($P<0.05$) in copper accumulation and depuration compared to that of tilapia in control water at each test. Copper elimination was slow with increasing elimination time and did not reach the control concentration after 96 hours of depuration in clean water. This indicated that copper was not completely eliminated from tilapia tissue after 96 hours of depuration test. Previous study revealed that rainbow trout (*Oncorhynchus mykiss*) from all Cu treatments and all sampling periods contained significantly higher ($P<0.05$) tissue Cu concentrations than controls (Hansen *et al.*, 2001).

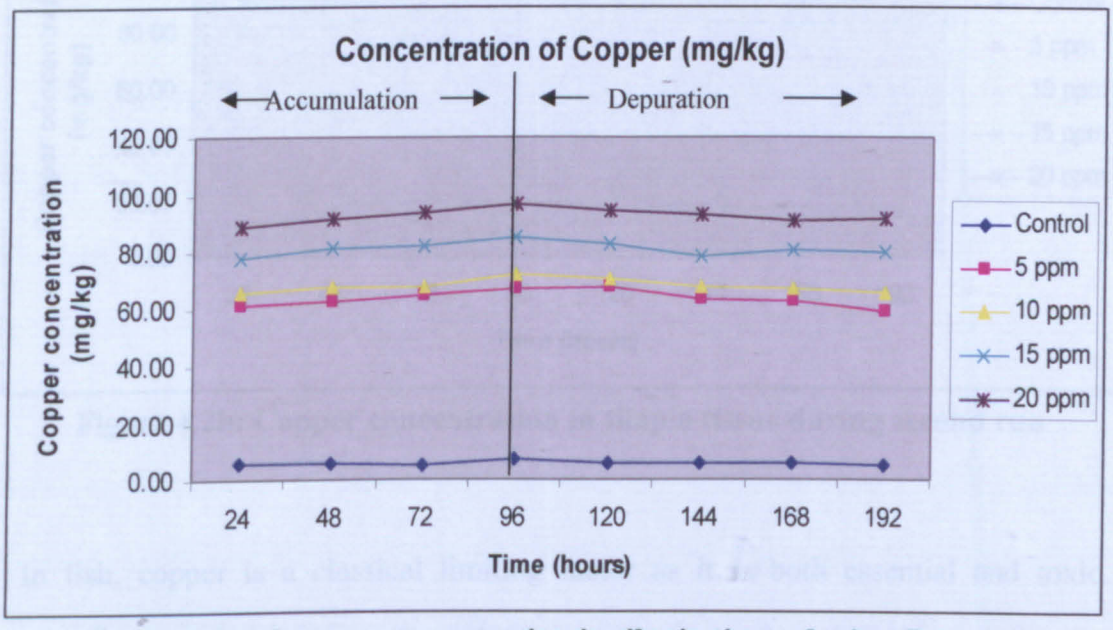


Figure 4.2a: Copper concentration in tilapia tissue during first run

Mean copper concentration measured in tilapia during the second round of test increased for all concentrations of exposure (Figure 4.2b). The mean measured copper concentration (with standard deviation, SE) for 0.0 ppm, 5.0 ppm, 10.0 ppm, 15.0 ppm and 20.0 ppm Cu exposures were 17.44 mg/kg (2.98), 72.23 mg/kg (2.96), 84.90 mg/kg (3.75), 84.75 mg/kg (3.24), and 93.42 mg/kg (3.04), respectively. Figure 4.2b showed that the concentrations of copper in tissues of tilapia increased with increasing exposure time. Statistical analyses indicated significant differences ($P<0.05$) in copper accumulation and depuration compared to that of tilapia in control water at each test during accumulation and depuration. Copper elimination was slow with increasing elimination time. From Figure 4.2b, it can be observed that the concentrations of copper did not reach the control concentration after 96 hours of depuration test in clean water.

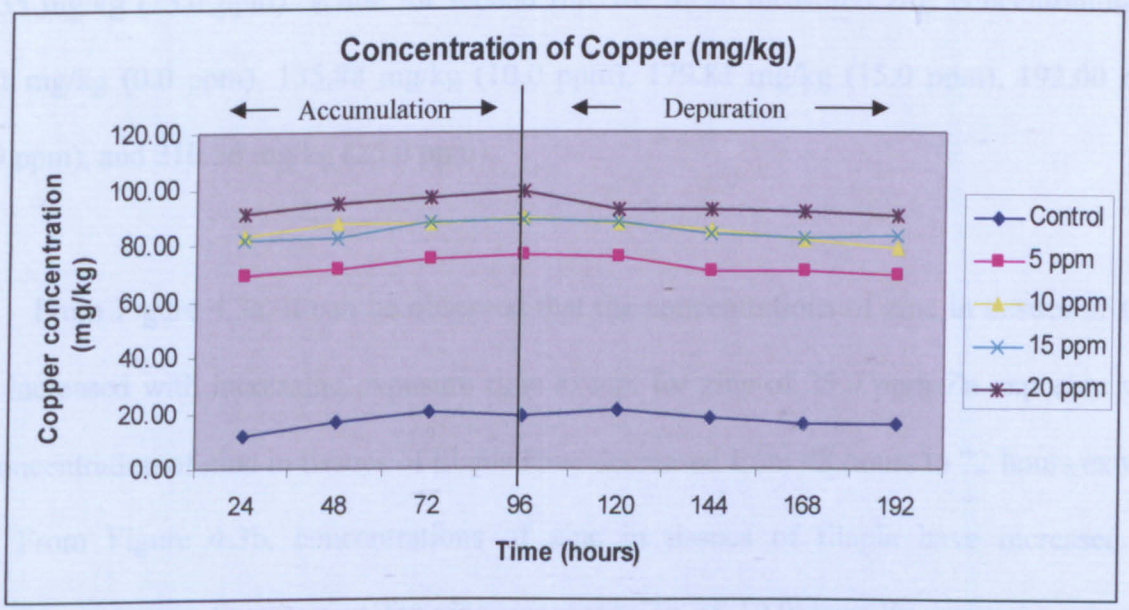


Figure 4.2b: Copper concentration in tilapia tissue during second run

In fish, copper is a classical limiting factor as it is both essential and toxic. As a micronutrient, it is necessary for haemaglobin synthesis and a component of Cytochrome oxidase (Benneth *et al.*, 1995). In this study, the fish exposed to copper were observed to be highly irritable and displayed frenzied swimming when approached, they swam upside down,

and their bodies were covered with thick mucus. These observations were similar to those of Oronsaye and Ogbebo (1995) who worked with adult *C. gariepinus* exposed to copper in soft water. The chemical analyses of tissues showed the accumulation of copper in fish tissues as fish exposed to high copper concentrations contained more copper than the control.

4.3 Zinc Accumulation and Depuration Test

Figure 4.3a and 4.3b represents the concentrations of zinc in tissues of tilapia at two different round of tests. The mean measured concentration of zinc was higher during first run of test compared to second test. Mean measured zinc concentration for first run were 41.85 mg/kg (0.0 ppm), 185.08 mg/kg (10.0 ppm), 205.96 mg/kg (15.0 ppm), 226.38 mg/kg (20.0 ppm), and 261.35 mg/kg (25.0 ppm). While for second run, the mean measured zinc concentration were 40.21 mg/kg (0.0 ppm), 135.88 mg/kg (10.0 ppm), 179.81 mg/kg (15.0 ppm), 192.00 mg/kg (20.0 ppm), and 210.56 mg/kg (25.0 ppm).

From Figure 4.3a, it can be observed that the concentrations of zinc in tissues of tilapia have increased with increasing exposure time except for zinc of 25.0 ppm Zn exposure where the concentration of zinc in tissues of tilapia have decreased from 48 hours to 72 hours exposure time. From Figure 4.3b, concentrations of zinc in tissues of tilapia have increased with increasing exposure time except for zinc concentration of 10.0 ppm Zn exposure where the concentrations of zinc in tissues of tilapia have decreased from 48h to 72h exposure time. Significant differences ($P<0.05$) was found for zinc accumulation and depuration compared to that of tilapia in control water at each test. Zinc elimination was slow with increasing elimination time and did not reach control concentration after 96 hours of depuration in clean water. The concentrations of zinc in tilapia tissues at 192 hours was higher than the

concentrations of zinc in tilapia tissues at 24 hours for all the zinc exposure samples. Spiked concentrations of zinc were retained in the tissues of zinc and no depuration took place. The differences in elimination of zinc probably related to the differences in regulation of zinc in tilapia. This results showed that tilapia was unable to regulate zinc as effectively.

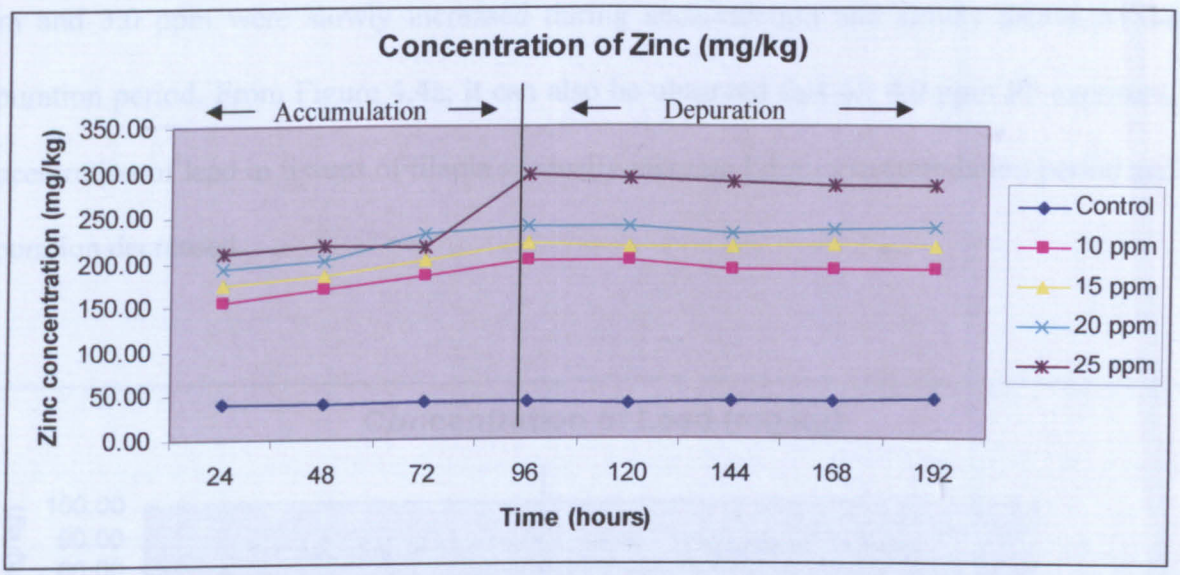


Figure 4.3a: Zinc concentration in tilapia tissue during first run

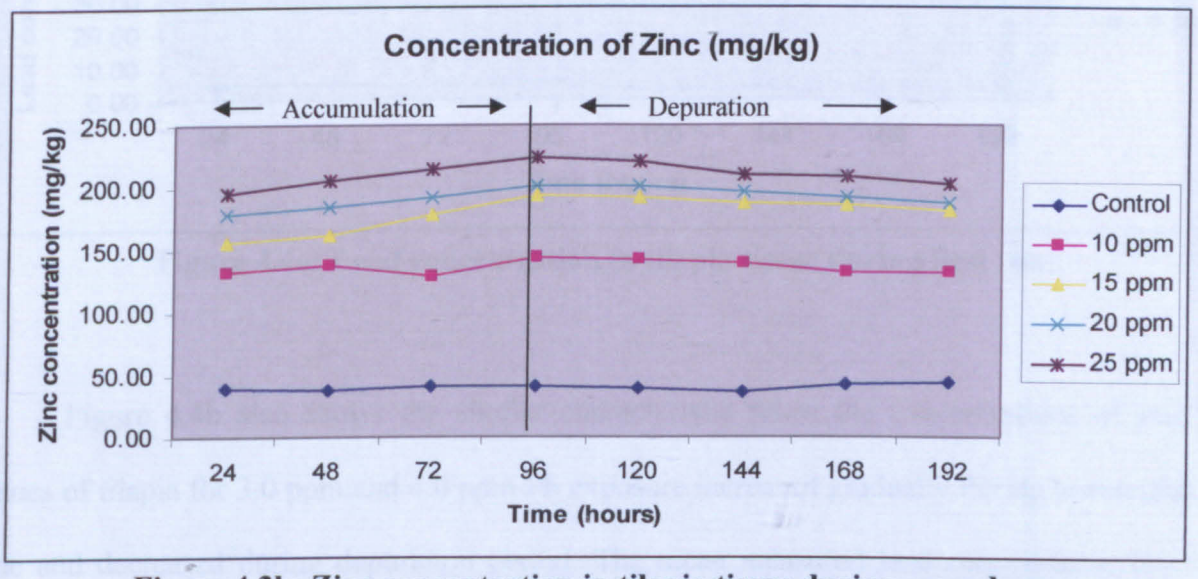


Figure 4.3b: Zinc concentration in tilapia tissue during second run

4.4 Lead Accumulation and Depuration Test

Figure 4.4a represents the concentrations of lead in tissues of tilapia for 0.0 ppm, 1.0 ppm, 2.0 ppm, 3.0 ppm and 4.0 ppm lead exposure. Similar accumulation and depuration trend can be observed in Figure 4.4b, where the concentrations of lead in tissues of tilapia for 1.0 ppm, 2.0 ppm and 3.0 ppm were slowly increased during accumulation and slowly decreased during depuration period. From Figure 4.4a, it can also be observed that for 4.0 ppm Pb exposure, the concentration of lead in tissues of tilapia gradually increased during accumulation period and the depuration decreased.

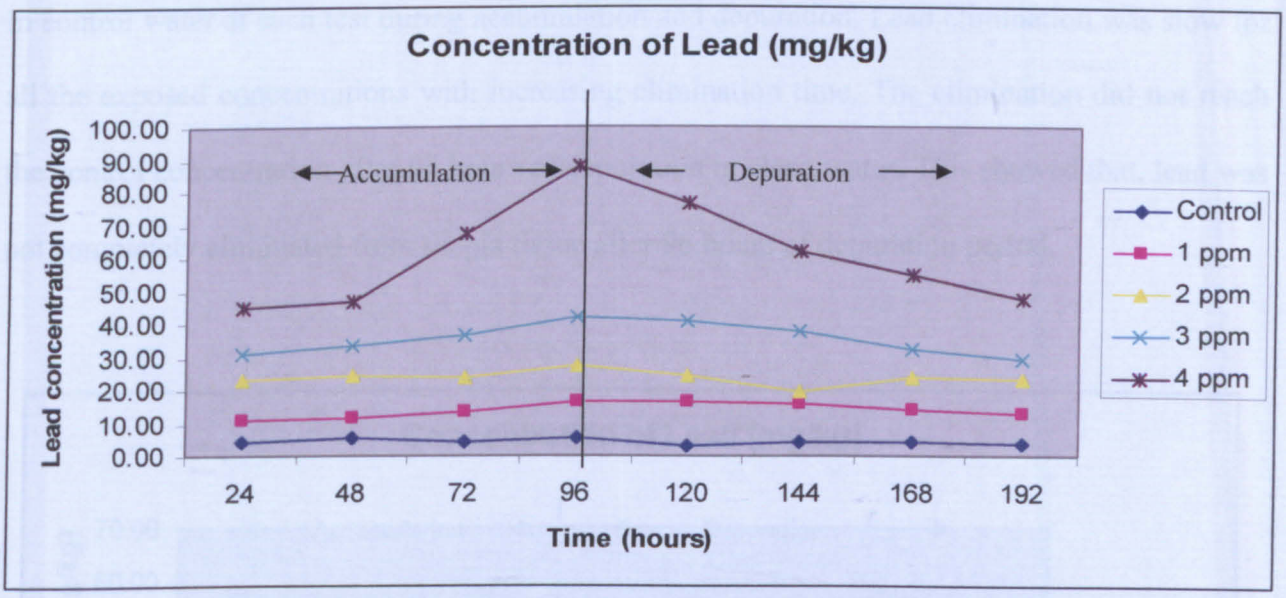


Figure 4.4a: Lead concentration in tilapia tissue during first run

Figure 4.4b also shows the similar characteristic when the concentrations of lead in tissues of tilapia for 3.0 ppm and 4.0 ppm Pb exposure increased gradually during accumulation time and decreased during depuration period. The mean measured lead concentration for 0.0 ppm, 1.0 ppm, 2.0 ppm, 3.0 ppm and 4.0 ppm Pb exposures were higher during first run of the test compared to the mean measured concentrations during second test. The mean measured

concentrations for first run were 4.38 ± 0.83 mg/kg for 0.0 ppm, 13.88 ± 2.12 mg/kg for 1.0 ppm, 23.54 ± 2.24 mg/kg for 2.0 ppm, 35.19 ± 4.65 mg/kg for 3.0 ppm, and 60.73 ± 15.50 mg/kg for 4.0 ppm while the mean measured concentration for second run were 1.50 ± 0.31 mg/kg for 0.0 ppm, 9.85 ± 2.55 mg/kg for 1.0 ppm, 22.27 ± 3.34 mg/kg for 2.0 ppm, 35.65 ± 4.34 mg/kg for 3.0 ppm, and 54.44 ± 9.66 mg/kg for 4.0 ppm. Figure 4.4a and Figure 4.4b also shows that the concentration of lead in tissues of tilapia increased with increasing exposure time except for 2.0 ppm lead exposure for first test and 4 ppm Pb exposure for second test where the concentrations of lead in tissues of tilapia have decreased from 48 hours to 72 hours exposure time and have decreased from 24 hours to 72 hours exposure time. Statistical analyses indicated significant differences ($P<0.05$) in lead accumulation and depuration compared to that of tilapia in control water at each test during accumulation and depuration. Lead elimination was slow for all the exposed concentrations with increasing elimination time. The elimination did not reach the control concentration after 96 hours of depuration in clean water. This showed that, lead was not completely eliminated from tilapia tissue after 96 hours of depuration period.

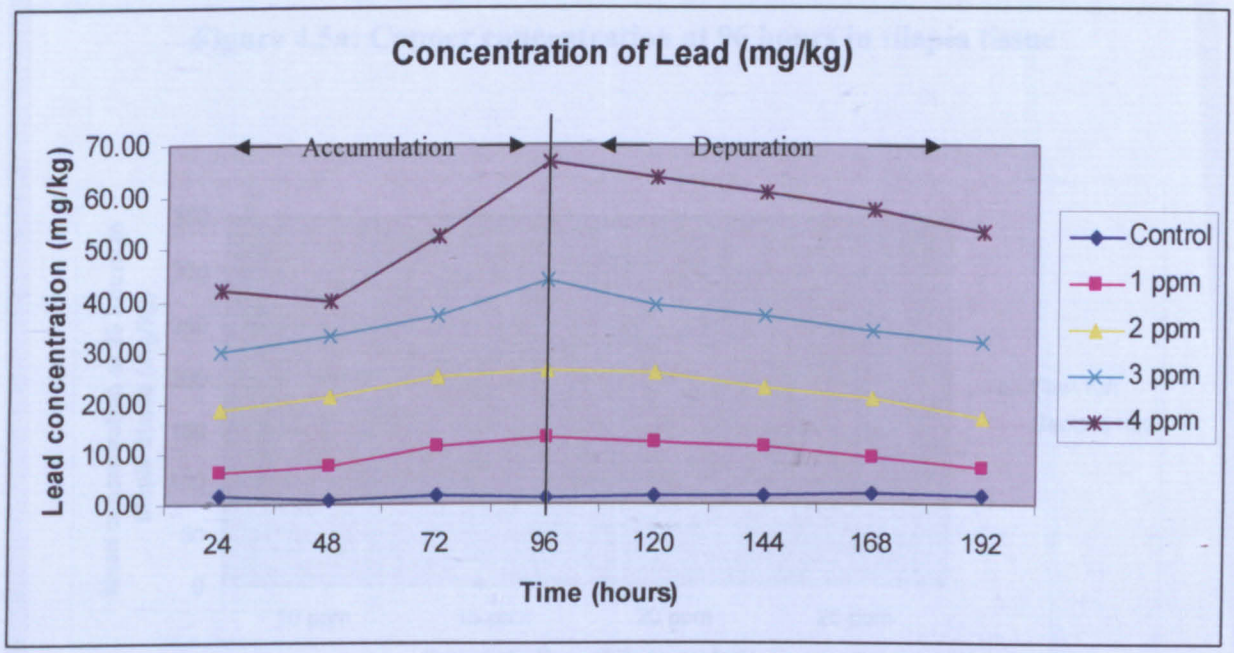


Figure 4.4b: Lead concentration in tilapia tissue during second run

4.5 Uptake of metals at 96 Hours in Tilapia Tissue

Figure 4.5a, 4.5b and 4.5c shows the accumulation of metals at 96 hours in tilapia tissues versus metal concentrations in water. It can be observed that there are direct accumulation of copper, zinc and lead by tilapia proportional to the concentration of copper, zinc and lead in water except for the copper exposure (second run). The mean copper concentrations for 15.0 ppm Cu exposure were slightly lower than the mean copper concentration for 10.0 ppm Cu exposure.

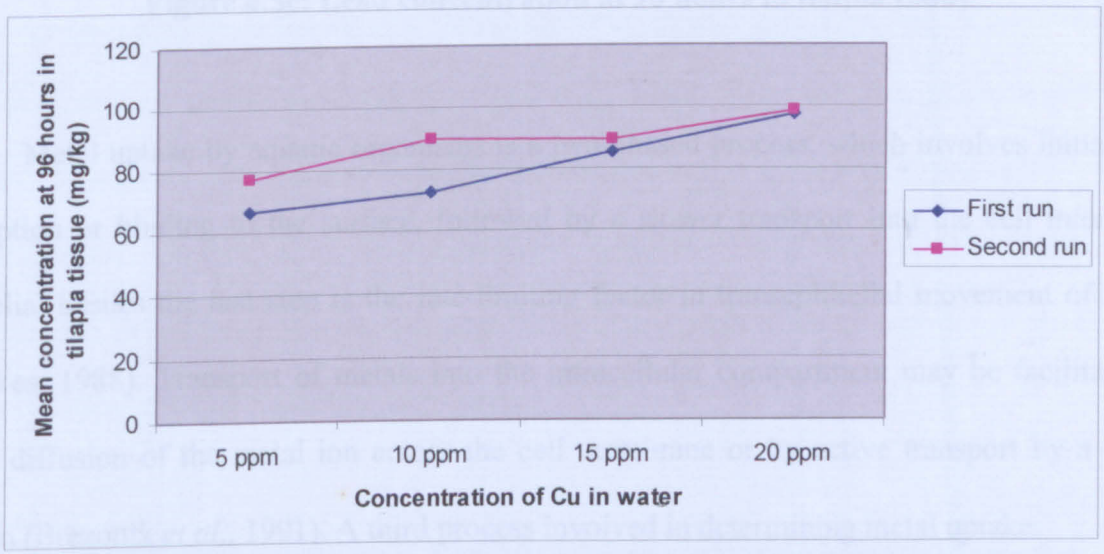


Figure 4.5a: Copper concentration at 96 hours in tilapia tissue

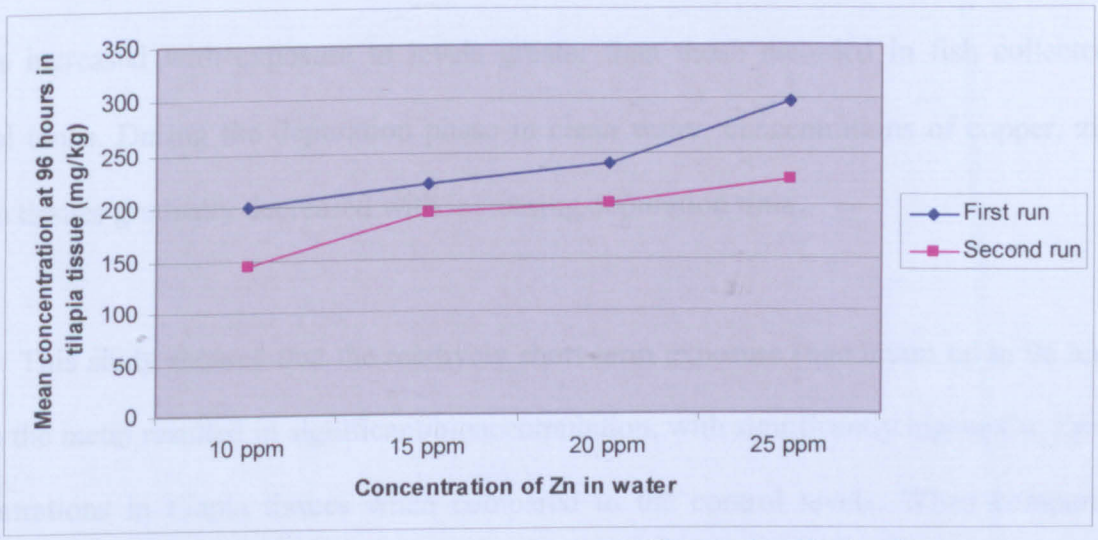


Figure 4.5b: Zinc concentration at 96 hours in tilapia tissue

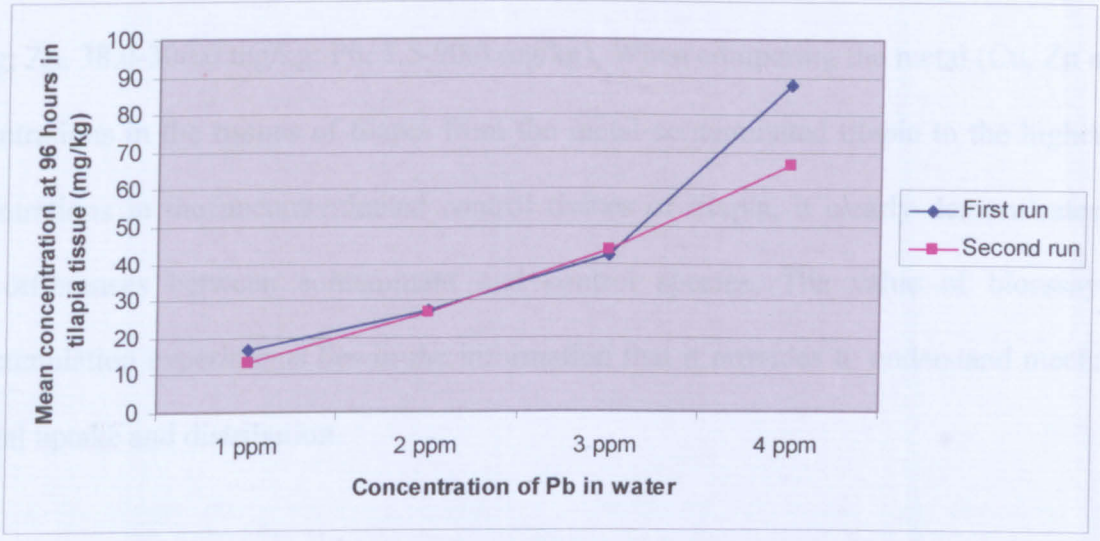


Figure 4.5c: Lead concentration at 96 hours in tilapia tissue

Metal uptake by aquatic organisms is a two-phased process, which involves initial rapid adsorption or binding to the surface, followed by a slower transport into the cell interior. In epithelial tissues the last step is the rate-limiting factor in transepithelial movement of metals (Foulkes, 1988). Transport of metals into the intracellular compartment may be facilitated by either diffusion of the metal ion across the cell membrane or by active transport by a carrier protein (Brezonik *et al.*, 1991). A third process involved in determining metal uptake.

As expected, during the accumulation test, concentrations of copper, zinc and lead in tissues increased with exposure to levels greater than those recorded in fish collected from control tanks. During the depuration phase in clean water, concentrations of copper, zinc and lead in tissues gradually decreased with increasing depuration time.

This study showed that the relatively short-term exposure (maximum up to 96 hours) of fish to the metal resulted in significant bioaccumulation, with significantly higher Cu, Zn and Pb concentrations in tilapia tissues when compared to the control levels. When comparing the bioassay bioaccumulation results to the actual metal bioaccumulation in tilapia, it was evident

that metal (Cu, Zn and Pb) concentrations were all within the different range (Cu, 6.0- 100.0 mg/kg; Zn, 38.0-300.0 mg/kg; Pb, 1.5-90.0 mg/kg). When comparing the metal (Cu, Zn and Pb) concentrations in the tissues of tilapia from the metal-contaminated tilapia to the higher metal concentrations in the uncontaminated control tissues of tilapia, it clearly demonstrates metal level differences between contaminant and control species. The value of bioassay-based bioaccumulation experiments lies in the information that it provides to understand mechanisms of metal uptake and distribution.

In the metal experiments, the chemicals (Cu, Pb and Zn), were eliminated slowly and most of the result of metal concentration did not reach the control concentrations after 96 hours of depuration period. The differences in elimination of metal (Cu, Zn and Pb) probably related to the differences regulation of these elements in tilapia. Mortality of tilapia fish during accumulation and depuration are less than 50%. There were small number of death occurred during the accumulation and depuration test.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

In order to understand the effect of pollutants on freshwater fish it is necessary to explain their mechanisms of accumulation and elimination, as well as relationship between body concentration of pollutant and the observed toxicity.

In the present study, the accumulation and elimination of copper, zinc and lead in tilapia was investigated. During metal exposures, tilapia tissue concentration of copper, zinc and lead increased with exposure time. Copper, zinc and lead elimination are rapid and some of them are slow with depuration period. The direct accumulation of Cu, Zn and Pb by tilapia *(Oreochromis* spp.*)* was proportional to the concentration of Cu, Zn and Pb in water. Tilapia was found to be a useful and continuous biomonitor of heavy metals, providing information on past and present environmental status of the water body.

As for future studies, it is recommended to take into consideration of other parameters such as conductivity, dissolved oxygen, total hardness, alkalinity, total organic carbon (TOC), total ammonia and other heavy metals such as As, Hg and Cd. The experiment frequency could also be extended to longer period in order to monitor the metal accumulations pattern in tilapia *(Oreochromis* spp.*)*. Besides that, other parts or organs such as gills, liver and gonad in the tilapia *(Oreochromis* spp.*)* can be also analyzed.

REFERENCES

Abel, P. D., and Papoutsouglou, S. E., 1986. Lethal toxicity of cadmium to Cyprinus carpio and Tlapia aurea. *Bull. Environ. Cont. Toxicol.* **37**, 382-386.

Alabastar, J. C., and Lloyd, R., 1982. *Copper in water quality, Criteria for freshwater fish.* Butterworths, London. 188-216.

Benneth, W. A., Sosa, A., and Beitinger, T. L., 1995. Oxygen tolerance of fathead Minnows previously exposed to copper. *J. Environ Cont and Toxicol.* **55**, 517-524.

Boudou, A., and Ribeyre, F., 1989. Fundamental Concepts and Methodologies. *Aquat Ecotox.* **2**, 332-337.

Brezonik, P., King, S., and and Mach, C., 199. The influence of water chemistry on trace metal bioavailability and toxicity to aquatic organisms. In: MC Newman and AW McIntosh (eds.) *Metal Ecotoxicology: Concepts and Applications*, Lewis Publishers Inc., Michigan. 1-26.

Canl, M., and Furness, R., 1993. Toxicity of heavy metals dissolved in sea water and influences of sex and size on metal accumulation and tissue distribution in the Norwey lobster Nephrops norvegicus. *Marine Environ. Res.* **36**, 217-236.

Carpené, E., Cattani, O., Serrazanetti, P., Fedrizzi, G., and Cortesi, P., 1990. Zinc and copper in fish from natural waters and rearing ponds in Northern Italy. *J. Fish Biol.* **37**, 293-299.

Copperinfo, 2001. Copper and Aquatic life. International Copper Association. http://www.copperinfo.com/health/aquatic.shtml. (Accessed date: 9 October 2006)

Daziel, J., and Baker, C., 1983. Analytical methods for measuring metals by atomic absorption spectrometry. *FAO. Fish. Tech.* **212**, 14-21.

FAO/SIDA, 1993. Manual of Methods in Aquatic Environmental Research, part 9. Analysis of Metals and Organochlorines in Fish. *FAO Fish Tech.* **212**, 25-33.

Foulkes, C., 1988. On the mechanism of transfer of heavy metals across cell membranes. *Toxicol.* **52**, 263-272.

Gatlin, D. M., and Wilson, R. P., 1983. Dietary zinc requirement of fingerling channel catfish. *J. Nutr.* **113**, 630-635.

Guven, K., Özbay, C., Ünlu, E. and Satar, A., 1999. Acute Lethal Toxicity and Accumulation of Copper in Grammarus pulex (L.) (Amphipoda). Tr. *J. Biol.* **23**, 513-521.

Hakanson, L., 1984. Metals in fish and sediment from the River Kolbacksan water system, Sweden. *Arch. Hydrobiol.* **101**, 373-400.

Hall, J. A., and Golding, L. A., 1998. Standard methods for whole effluent toxicity testing: development and application. Report no. MFE80205. NIWA report for the ministry for the Environment, Wellington, New Zealand. 2-22.

Handy, R.D., and Shaw, B.J., 2006. Dietary copper exposure and recovery in Nile tilapia, *Oreochromis niloticus. J. Aquat. Toxicol.* **76**, 111-121.

Hansen, J.A., Lipton, J., Welsh, P.G., 2001. Relative sensitivity of bull trout and raibow trout to acute copper toxicity. *J. Aquat. Toxicol.* **58**, 175-188.

Hawkes, S. J., 1997. "What Is a Heavy Metal?" *J. Chem. Edu.* **74**, 1374.

Hodson, P. V., 1976. δ-amino levulinic acid dehydratase activity of fish blood as an indicator of a harmful exposure to lead. *J. Fish. Res. Board Can.* **33**, 268-271.

Hodson, P. V., Blunt, B. R., and Spry, D. J., 1978. Chronic toxicity of waterborne and dietary lead to rainbow trout (*Salmo gairdneri*) in lake Ontario water. *Wat Res.* **12**, 869-878.

Hodson, P. V., Blunt, B. R., Jensen, D., and Morgan, S., 1979. Effect of fish age on predicted and observed chronic toxicity of lead to rainbow trout in lake Ontario water. *J. Great Lakes Res.* **5**, 84-89.

Hodson, P. V., Blunt, B. R., and Slinger, S.J., 1980. Effect of dietary ascorbic acid on chronic lead toxicity to young rainbow trout (*Salmo gairdneri*). *Can. J. Fish. Aquat. Sci.* **37**, 170-176.

Hodson, P. V., 1988. The effect of metal metabolism on uptake, disposition and toxicity in fish. *Aquat. Toxicol.* **11**, 3-18.

Hogstrand, C., and Wood, C. M., 1996. The physiology and toxicology of zinc in fish. In *Toxicology of Aquatic Pollution* (ed. E. W. Taylor). 61-84.

Huet, M., 1994. *Text Book of Fish culture: Breeding and cultivation of fish*, 2nd edn. University Press. Cambridge. 54-63.

John, H. D., 2002. "Heavy metals"- A meaningless term? (IUPAC Technical report). *Pure Appl. Chem.***74**, 793–807.

Kalay, M., and Canl, M., 2000. Elimination of essential (Cu, Zn) and nonessential (Cd, Pb) metals from tissue of a freshwater fish Tilapia zilli. *Turkish J. Zool.* **24**, 429-436.

Kumar, A., and Mathur, R. P., 1991. Bioaccumulation kinetics and organ distribution of lead in a fresh water teleost, *Colisa fasciatus. Environ. Tech.* **12**, 731-735.

Lacher, T. E., and Goldstein, M. I., 1997. Tropical ecotoxicology: status and needs. *Environ. Toxicol. Chem.* **16**, 100-11.

Lanno, R. P., Slinger, S. J., and Hilton, J. W., 1985. Maximum tolerable and toxicity levels of dietary copper in rainbow-trout (*Salmo gairdneri* Richardson). *Aquaculture* **49**, 257-268.

Larsson, A., Haux, C., and Sjöbeck, M., 1985. Fish pshysiology and metal pollution: results and experiences from laboratory and field studies. *Ecotox. Environ. Safety.* **9**, 250-281.

Laurén, D. J., and McDonald, D. G., 1985. Effects of copper on branchial ionoregulation in the rainbow trout, *Salmo gairdneri* Richardson modulation by water hardness and pH. *J. Comp. Physiol. B* **155**, 635-644.

Mair, J. F. and Roberts, R. J., 1988. Recent advances in aquaculture. *Aquaculture. 23*, 407-409.

Mansour, S. A., and Sidky, M. M., 2002. Ecotoxicological studies. 3. Heavy metals contaminating water and fish from fayoum Governorate, Egypt. *Food chem.* **78**, 15-22.

Mazon, A. F., and Fernandes, M. N., 1999. Toxicity and differential tissue accumulation of copper in the tropical freshwater fish, *Prochilodus scrofa* (Prochilodontidae). *Bull. Environ. Cont. Toxicol.* **63**, 797-804.

McCall, K. A., Huang, C. C., and Fierke, C., 2000. Function and mechanism of zinc metalloenzymes. *J. Nutr.* **130**, 1437-1446.

Moore, B. K., Mitchell, A. J., Griffin, B. R. and Huffman, G. L., 1984. Parasites and Diseases of pond fishes. Third report of the fish Farmers: US fish and Wildlife services, Washington, D.C. 53-59.

Nicolas, R. B., Paul, A. W., and Chris, N. G., 2003. Nutritive metal update in teleost fish. *J. Experimental Bio.* **206**, 11-23.

Ogino, C., and Yang, G.Y., 1978. Requirement of rainbow trout for dietary zinc. *Bull. Soc. Sci. Fish.* **44**, 1015-1018.

Olaifa, F. E., Olaifa, A. K., and Onwude, T. E., 2004. Lethal and sub-lethal efects of copper to the African Catfish *(Clarias gariepinus)* Juveniles. *African J. Biol Res.* **7**, 65-70.

Oliveira, C. A., Fanta, E., Turcattin, N. M., Cardoso, R. J., and Carvalho, C. S., 1996. Lethal effects of inorganic mercury on cells and tissues of *Trichomycterus brasiliensis* (Pisces, Siluroidei). *Biocell.* **20**, 171-178.

Oliveira, C. A., Pelletier, E., Pfeiffer, W. C., and Rouleau, C., 2000. Comparative uptake, bioaccumulation, and gill damages of inorganic mercury in tropical and Nordic freshwater fish. *Environ. Res.* **83**, 286-292.

Oronsaye, J. A. O., and Ogbebo, F. E., 1995. The acute toxicity of copper to *Clarias gariepinus* in soft water. *J. Aquat Sci.* **10**, 19-23.

Oronsaye, J. A. O., and Ogunbor, E. O., 1998. Toxicity of Copper to *Oreochromis niloticus* fingerling in Ikpoba River water. *Indian J. Animal Sci*, **68 (9)**, 1001-1003.

Powell, S. R., 2000. The antioxidant properties of zinc. *J. Nutr.* **130**, 1447- 1454.

Richard D. H., and Benjamin J. S., 2005. Dietary copper exposure and recovery in Nile tilapia, Oreochromis niloticus. *Aquat Toxicol.* **76**, 111-121.

Salmeron-flores, P., Melendez-Camargo, M.E., and martinez-Tabche, L., 1990. Efecto hepatotóxico y nefrotóxico del plomo sobre la tilapia (*Sarotherodon aureus*). *An. Esc. Nac. Cienc. Biol. Mex.* **33**, 147-156.

Spry, D. J., and Wood, C. M., 1985. Ion flux rates, acid–base status, and blood gases in rainbow trout, *Salmo gairdneri*, exposed to toxic zinc in natural soft water. *Can. J. Fish. Aquat. Sci.* **42**, 1332-1341.

Suhaimi-Othman, M. and Pascoe, D., 2007. Bioconcentration and depuration of Copper, Cadmium and Zinc mixtures by the freshwater Amphipod *Hyalella azteca*. *J. Ecotox. And Environ. Safety.* **66**, 29-35.

Suhendrayatna., Akira O., Tsunenori N., and Shineru M., 2001. Studies on the accumulation and transformation of arsenic in freshwater organisms and tilapia mossambica. *Chemosphere*. **46**, 325-331.

Thomas, P., and Michael, M., 1999. Tilapia Life History and Biology. *Southern Regional Aquaculture Center (SRAC)*. **283**, 37-45.

Trewavas, E., 1983. Tilapiine fishes of the genera Sarotherodon, Oreochromis and Danakilia. London : British Museum (Natural History). 23-26.

Ünlü, E., and Gümgüm, B., 1993. Concentrations of copper and zinc in fish and sediments from the Tigris river in Turkey. *Chemosphere*. **26**, 2055-2061.

Vallee, B. L., and Falchuk, K. H., 1993. The biochemical basis of zinc physiology. *Physiol. Rev.* **73**, 79-118.

Vera, M. P., Pocsidio, G. N., De-veram, P., 1998., Potential protective effect of Calcium Carbonate as liming against copper toxicity in the African tilapia Oreochromis mossambius. *Sci. Total Environ.* **214**, 193-202.

Wang, W.X., and Zhang, L., 2006. Effects of Zn pre-exposure on Cd and Zn bioaccumulation and metallothionein levels in two species of marine fish. *J. Aquat. Toxicol.* **73**, 353-369.

Watanabe, W. O., Ellingson, L. J., and Wicklund, R. I., 1987. The effects of salinity on growth. Food consumption and conversion in Juvenile, Monosex mal Florida red tilapia. In *the 2nd Int. Symp. on tilapia in Aquacult*, ed. R. S. V. Dullin. T. Bhukaswas. K. Tonguthai And J. L. Maclean. ICLARM. Thailand. 32-52.

Watanabe, T., Kiron, V., and Satoh, S., 1997. Trace minerals in fish nutrition. *Aquaculture* **151**, 185-207.

Weis, J. S., and Weis, P., 1989. Effect of environmental pollutants on early fish development. *Rev. Aquat. Sci.* **1**, 45-73.

Wepener, V., Vuren, V., and Preez, D., 2001. Uptake and distribution of a copper, iron and zinc mixture in gill, liver and plasma of a freshwater teleost, *Tilapia sparrmanii*. *Wat Res.* **27**, 99-108.

WHO, 1995. *Environmental health criteria 85: Lead – environmental aspects.* World Health Organization, Geneva. 17-25.

Wong, M. H., and Chiu, S. T., 1993. Feasibility studies on the use of sewage sludge as supplementary feed for rearing *tilapia. Environ.Tech.* **20**, 375-384.

Wong, M. H., Hung, K. M., and Chiu, S. T., 1996. Sludge-growth algae for culturing aquatic organisms. Part II, Sludge-groeth algae as foods for aquatic organism. *Environ. Manage.* **20**, 375-384.

Wong, M. H., Zhou, H. Y., Cheung, R. y., and Chan, K. M., 1998. Metal concentrations in sediments and tilapia collected from inland waters of Hong Kong. *Wat. Res.* **32**, 231-240.

Woodward D. F., Farag, A. M., Bergman, H. L., Delonay, A. J., Little, E. E., Smith, C. E., and Barrows, F. T., 1995. Metals contaminated benthic invertebrates in the Clark Fork River, Montana: effects on age-0 brown trout and rainbow trout. *Can. J. Fish. Aquat. Sci.* **52**, 1994-2004.

Yap, C. K., Ismail, A., Tan, S. G., and Abdul Rahim, I., 2003. Can the Shell of the Green-lipped Mussel Perna viridis (Linnaeus) from the west Coast of Peninsular Malaysia be a potential Biomonitoring Material for Cd, Pb and Zn?. *Estuar. Coast. Sci.* **12**, 34-40.

Yilmaz, A. B., 2005. Comparison of heavy metal levels of grey mullet (Mugil cephalusL.) and sea bream (Sparus aurataL.) caught in Skenderun Bay (Turkey). *Turk J. Anim Sci.* **29**, 257-262.

APPENDIX A
Experimental Data

Appendix A-1

Table 1: Raw Data From AAS: Tilapia Samples

1.1 Copper Concentration (first run)

Sample		Concentration of Copper (ppm)							
		Acc 1 (24h)	Acc 2 (48h)	Acc 3 (72h)	Acc 4 (96h)	Dep 1 (120h)	Dep 2 (144h)	Dep 3 (168h)	Dep 4 (192h)
Control	1	0.12±0.002	0.13±0.001	0.10±0.002	0.15±0.001	0.12±0.002	0.09±0.003	0.11±0.002	0.13±0.003
	2	0.11±0.003	0.11±0.002	0.10±0.001	0.15±0.002	0.12±0.004	0.12±0.002	0.11±0.005	0.09±0.002
	3	0.13±0.002	0.13±0.001	0.11±0.001	0.14±0.009	0.13±0.003	0.12±0.004	0.12±0.001	0.10±0.007
5 ppm	1	1.21±0.001	1.26±0.023	1.29±0.012	1.35±0.003	1.33±0.002	1.25±0.002	1.25±0.005	1.19±0.001
	2	1.21±0.004	1.24±0.001	1.29±0.003	1.33±0.001	1.36±0.007	1.27±0.002	1.28±0.006	1.18±0.002
	3	1.23±0.003	1.24±0.006	1.30±0.001	1.32±0.004	1.39±0.001	1.29±0012	1.23±0.000	1.16±0.001
10 ppm	1	1.30±0.003	1.37±0.001	1.36±0.013	1.43±0.003	1.40±0.001	1.36±0.013	1.35±0.004	1.32±0.001
	2	1.32±0.004	1.33±0.001	1.32±0.003	1.45±0.011	1.42±0.003	1.36±0.005	1.33±0.006	1.30±0.014
	3	1.31±0.001	1.37±0.004	1.38±0.001	1.45±0.004	1.41±0.003	1.35±0.001	1.32±0.005	1.29±0.003
15 ppm	1	1.57±0.016	1.63±0.003	1.65±0.001	1.71±0.003	1.67±0.003	1.59±0.001	1.62±0.004	1.59±0.003
	2	1.56±0.002	1.62±0.001	1.64±0.001	1.71±0.002	1.68±0.004	1.57±0.002	1.60±0.003	1.62±0.004
	3	1.55±0.001	1.63±0.003	1.64±0.002	1.70±0.004	1.63±0.001	1.56±0.004	1.61±0.002	1.58±0.004
20 ppm	1	1.85±0.003	1.84±0.001	1.88±0.003	1.96±0.006	1.90±0.007	1.87±0.006	1.87±0.005	1.84±0.007
	2	1.70±0.003	1.84±0.001	1.86±0.007	1.93±0.007	1.90±0.006	1.89±0.005	1.79±0.006	1.83±0.001
	3	1.78±0.003	1.82±0.003	1.90±0.003	1.93±0.003	1.88±0.003	1.83±0.001	1.80±0.003	1.82±0.001

1.2 Copper Concenration (second run)

Sample		Concentration of Copper (ppm)							
		Acc 1 (24h)	Acc 2 (48h)	Acc 3 (72h)	Acc 4 (96h)	Dep 1 (120h)	Dep 2 (144h)	Dep 3 (168h)	Dep 4 (192h)
Control	1	0.24±0.002	0.32±0.001	0.40±0.002	0.46±0.005	0.43±0.002	0.38±0.001	0.31±0.003	0.32±0.001
	2	0.23±0.002	0.34±0.001	0.41±0.001	0.33±0.003	0.40±0.002	0.37±0.002	0.33±0.001	0.32±0.002
	3	0.23±0.001	0.33±0.002	0.43±0.002	0.35±0.002	0.42±0.003	0.35±0.003	0.33±0.001	0.34±0.002
5 ppm	1	1.39±0.003	1.44±0.002	1.49±0.002	1.53±0.002	1.53±0.001	1.40±0.002	1.42±0.002	1.40±0.003
	2	1.38±0.003	1.43±0.002	1.49±0.002	1.55±0.001	1.50±0.004	1.43±0.003	1.42±0.001	1.38±0.001
	3	1.37±0.001	1.41±0.003	1.50±0.002	1.52±0.004	1.50±0.003	1.41±0.004	1.41±0.003	1.37±0.002
10 ppm	1	1.65±0.003	1.75±0.001	1.76±0.004	1.80±0.004	1.74±0.002	1.71±0.002	1.64±0.004	1.60±0.003
	2	1.66±0.001	1.74±0.002	1.74±0.001	1.79±0.003	1.74±0.001	1.70±0.002	1.63±0.001	1.55±0.002
	3	1.65±0.001	1.76±0.003	1.75±0.003	1.79±0.004	1.74±0.002	1.69±0.003	1.60±0.003	1.57±0.001
15 ppm	1	1.63±0.002	1.76±0.001	1.60±0.001	1.79±0.001	1.65±0.002	1.77±0.004	1.69±0.001	1.70±0.004
	2	1.65±0.002	1.77±0.001	1.63±0.004	1.77±0.001	1.63±0.002	1.74±0.001	1.65±0.001	1.67±0.002
	3	1.65±0.001	1.77±0.003	1.65±0.001	1.78±0.001	1.64±0.001	1.79±0.002	1.65±0.003	1.65±0.002
20 ppm	1	1.80±0.003	1.89±0.002	1.94±0.002	1.96±0.002	1.85±0.003	1.83±0.002	1.86±0.002	1.82±0.005
	2	1.83±0.001	1.88±0.003	1.93±0.001	1.98±0.002	1.84±0.002	1.83±0.004	1.84±0.003	1.80±0.002
	3	1.82±0.001	1.90±0.004	1.95±0.002	1.98±0.001	1.84±0.002	1.88±0.002	1.80±0.002	1.79±0.003

1.3 Zinc Concentration (first run)

Sample		Concentration of Zinc (ppm)							
		Acc 1 (24h)	Acc 2 (48h)	Acc 3 (72h)	Acc 4 (96h)	Dep 1 (120h)	Dep 2 (144h)	Dep 3 (168h)	Dep 4 (192h)
Control	1	0.80±0.055	0.86±0.072	0.95±0.051	0.85±0.062	0.85±0.070	0.91±0.098	0.84±0.051	0.92±0.081
	2	0.80±0.068	0.82±0.055	0.83±0.053	0.84±0.072	0.70±0.053	0.83±0.042	0.87±0.067	0.84±0.055
	3	0.81±0.062	0.80±0.075	0.73±0.084	0.85±0.057	0.88±0.064	0.83±0.055	0.80±0.063	0.88±0.070
10 ppm	1	3.10±0.067	3.38±0.042	3.64±0.072	4.02±0.067	4.00±0.068	3.83±0.088	3.82±0.033	3.79±0.051
	2	3.11±0.051	3.38±0.056	3.72±0.055	4.02±0.042	4.01±0.050	3.82±0.021	3.82±0.047	3.82±0.039
	3	3.08±0.051	3.36±0.068	3.65±0.051	4.00±0.047	4.03±0.099	3.84±0.068	3.81±0.042	3.79±0.086
15 ppm	1	3.50±0.049	3.71±0.023	4.03±0.042	4.44±0.099	4.32±0.018	4.30±0.033	4.35±0.043	4.32±0.024
	2	3.50±0.058	3.68±0.067	4.02±0.043	4.42±0.086	4.32±0.029	4.30±0.051	4.34±0.078	4.34±0.035
	3	3.49±0.057	3.68±0.067	4.04±0.056	4.43±0.045	4.33±0.054	4.32±0.021	4.36±0.066	4.32±0.050
20 ppm	1	3.89±0.019	4.04±0.045	4.62±0.078	4.80±0.033	4.79±0.013	4.60±0.047	4.73±0.025	4.76±0.058
	2	3.87±0.058	4.02±0.099	4.62±0.073	4.83±0.057	4.79±0.086	4.63±0.033	4.75±0.064	4.76±0.012
	3	3.87±0.045	4.01±0.037	4.61±0.024	4.76±0.044	4.80±0.039	4.63±0.022	4.70±0.038	4.78±0.049
25 ppm	1	4.21±0.042	4.37±0.059	4.31±0.018	5.94±0.051	5.88±0.068	5.76±0.057	5.67±0.042	5.69±0.067
	2	4.20±0.057	4.37±0.047	4.38±0.044	5.94±0.055	5.86±0.064	5.76±0.053	5.67±0.023	5.69±0.054
	3	4.23±0.011	4.33±0.043	4.35±0.056	5.92±0.051	5.83±0.035	5.75±0.049	5.67±0.045	5.67±0.032

1.4 Zinc Concentration (second run)

Sample		Concentration of Zinc (ppm)							
		Acc 1 (24h)	Acc 2 (48h)	Acc 3 (72h)	Acc 4 (96h)	Dep 1 (120h)	Dep 2 (144h)	Dep 3 (168h)	Dep 4 (192h)
Control	1	0.77±0.013	0.76±0.077	0.83±0.013	0.82±0.066	0.79±0.013	0.74±0.066	0.84±0.013	0.87±0.013
	2	0.77±0.005	0.77±0.028	0.82±0.013	0.82±0.005	0.77±0.021	0.73±0.005	0.86±0.025	0.88±0.031
	3	0.78±0.067	0.75±0.024	0.82±0.057	0.82±0.025	0.80±0.031	0.75±0.062	0.86±0.034	0.88±0.005
10 ppm	1	2.63±0.031	2.75±0.011	2.58±0.058	2.88±0.025	2.84±0.013	2.76±0.055	2.71±0.006	2.63±0.047
	2	2.65±0.025	2.75±0.005	2.60±0.061	2.88±0.013	2.85±0.005	2.78±0.034	2.60±0.005	2.64±0.035
	3	2.64±0.055	2.73±0.009	2.59±0.055	2.87±0.031	2.87±0.009	2.79±0.031	2.56±0.003	2.64±0.056
15 ppm	1	3.13±0.031	3.25±0.025	3.58±0.076	3.88±0.064	3.84±0.060	3.76±0.048	3.71±0.012	3.63±0.025
	2	3.13±0.045	3.23±0.013	3.57±0.077	3.86±0.007	3.84±0.025	3.77±0.044	3.71±0.013	3.65±0.042
	3	3.13±0.025	3.21±0.005	3.60±0.005	3.86±0.005	3.85±0.047	3.77±0.013	3.72±0.024	3.64±0.005
20 ppm	1	3.56±0.037	3.72±0.017	3.89±0.048	4.08±0.007	4.02±0.005	3.94±0.025	3.86±0.060	3.79±0.088
	2	3.58±0.027	3.74±0.019	3.83±0.031	4.00±0.060	4.02±0.009	3.92±0.034	3.86±0.069	3.77±0.056
	3	3.57±0.005	3.60±0.042	3.82±0.031	4.03±0.044	4.03±0.003	3.92±0.005	3.86±0.043	3.75±0.060
25 ppm	1	3.94±0.065	4.12±0.013	4.33±0.025	4.48±0.013	4.37±0.006	4.23±0.022	4.19±0.055	4.04±0.013
	2	3.93±0.060	4.12±0.018	4.30±0.057	4.49±0.043	4.39±0.005	4.20±0.025	4.16±0.031	4.05±0.057
	3	3.90±0.060	4.13±0.014	4.31±0.005	4.47±0.033	4.50±0.002	4.20±0.013	4.16±0.055	4.06±0.033

1.5 Lead Concentration (first run)

Sample		Concentration of Lead (ppm)							
		Acc 1 (24h)	Acc 2 (48h)	Acc 3 (72h)	Acc 4 (96h)	Dep 1 (120h)	Dep 2 (144h)	Dep 3 (168h)	Dep 4 (192h)
Control	1	0.09±0.082	0.10±0.047	0.08±0.055	0.11±0.045	0.08±0.078	0.09±0.041	0.12±0.041	0.07±0.023
	2	0.09±0.062	0.11±0.066	0.09±0.060	0.12±0.099	0.05±0.093	0.07±0.054	0.07±0.045	0.07±0.087
	3	0.08±0.088	0.12±0.078	0.07±0.034	0.11±0.067	0.07±0.066	0.08±0.093	0.08±0.080	0.08±0.045
1 ppm	1	0.22±0.075	0.24±0.070	0.27±0.041	0.34±0.023	0.33±0.078	0.32±0.023	0.30±0.069	0.27±0.065
	2	0.20±0.087	0.23±0.045	0.26±0.056	0.33±0.066	0.32±0.075	0.31±0.067	0.28±0.054	0.25±0.041
	3	0.23±0.044	0.23±0.056	0.26±0.023	0.31±0.045	0.32±0.045	0.33±0.079	0.26±0.097	0.25±0.093
2 ppm	1	0.46±0.093	0.49±0.023	0.43±0.047	0.55±0.093	0.50±0.088	0.40±0.099	0.48±0.049	0.49±0.045
	2	0.47±0.062	0.48±0.049	0.50±0.089	0.54±0.054	0.49±0.070	0.38±0.012	0.48±0.045	0.47±0.061
	3	0.44±0.041	0.48±0.068	0.49±0.044	0.55±0.045	0.46±0.049	0.37±0.093	0.47±0.089	0.43±0.020
3 ppm	1	0.63±0.080	0.68±0.071	0.79±0.077	0.84±0.089	0.81±0.071	0.75±0.067	0.64±0.054	0.61±0.041
	2	0.62±0.093	0.65±0.034	0.77±0.046	0.82±0.075	0.80±0.059	0.75±0.041	0.64±0.049	0.55±0.099
	3	0.60±0.071	0.66±0.088	0.77±0.041	0.86±0.041	0.81±0.039	0.75±0.045	0.66±0.053	0.57±0.055
4 ppm	1	0.89±0.060	0.98±0.047	1.42±0.094	1.74±0.043	1.53±0.028	1.24±0.021	1.07±0.049	0.93±0.071
	2	0.90±0.080	0.91±0.076	1.30±0.033	1.77±0.075	1.53±0.045	1.23±0.035	1.08±0.055	0.99±0.060
	3	0.90±0.041	0.90±0.043	1.30±0.023	1.75±0.033	1.52±0.052	1.23±0.078	1.12±0.067	0.92±0.051

1.6 Lead Concentration (second run)

Sample		Concentration of Lead (ppm)							
		Acc 1 (24h)	Acc 2 (48h)	Acc 3 (72h)	Acc 4 (96h)	Dep 1 (120h)	Dep 2 (144h)	Dep 3 (168h)	Dep 4 (192h)
Control	1	0.03±0.061	0.02±0.056	0.04±0.055	0.03±0.066	0.04±0.044	0.03±0.083	0.05±0.028	0.04±0.044
	2	0.03±0.032	0.02±0.067	0.03±0.045	0.02±0.060	0.02±0.059	0.03±0.077	0.03±0.052	0.03±0.037
	3	0.04±0.045	0.02±0.088	0.02±0.032	0.02±0.053	0.04±0.024	0.03±0.072	0.04±0.057	0.02±0.075
1 ppm	1	0.14±0.055	0.16±0.060	0.23±0.056	0.26±0.043	0.25±0.056	0.23±0.070	0.19±0.057	0.15±0.066
	2	0.13±0.045	0.15±0.057	0.23±0.033	0.25±0.066	0.25±0.067	0.21±0.060	0.18±0.023	0.15±0.033
	3	0.12±0.034	0.14±0.078	0.23±0.097	0.28±0.039	0.24±0.068	0.24±0.054	0.18±0.035	0.14±0.078
2 ppm	1	0.38±0.077	0.43±0.034	0.49±0.070	0.54±0.045	0.52±0.099	0.46±0.078	0.40±0.052	0.35±0.033
	2	0.38±0.057	0.42±0.057	0.50±0.030	0.53±0.051	0.51±0.088	0.46±0.084	0.42±0.081	0.33±0.049
	3	0.37±0.044	0.43±0.078	0.51±0.029	0.52±0.043	0.51±0.059	0.45±0.055	0.43±0.051	0.35±0.075
3 ppm	1	0.59±0.023	0.67±0.112	0.74±0.089	0.88±0.071	0.78±0.065	0.74±0.087	0.69±0.056	0.63±0.077
	2	0.61±0.014	0.65±0.072	0.74±0.030	0.86±0.072	0.80±0.098	0.74±0.089	0.68±0.019	0.64±0.070
	3	0.60±0.072	0.66±0.097	0.74±0.045	0.87±0.044	0.76±0.035	0.73±0.124	0.66±0.043	0.65±0.040
4 ppm	1	0.84±0.030	0.79±0.085	1.07±0.055	1.32±0.057	1.28±0.023	1.23±0.103	1.14±0.045	1.05±0.030
	2	0.84±0.035	0.80±0.099	1.03±0.051	1.33±0.067	1.28±0.034	1.22±0.060	1.13±0.056	1.06±0.034
	3	0.85±0.057	0.80±0.059	1.04±0.077	1.34±0,078	1.27±0.054	1.20±0.066	1.16±0.033	1.06±0.066

Appendix A-2

Table 2: Concentration of Heavy Metals

2.1 Copper Concentration (first run)

Sample		Concentration of Copper (mg/kg)							
		Acc 1 (24h)	Acc 2 (48h)	Acc 3 (72h)	Acc 4 (96h)	Dep 1 (120h)	Dep 2 (144h)	Dep 3 (168h)	Dep 4 (192h)
Control	1	6.0	6.5	5.0	7.5	6.0	4.5	5.5	6.5
	2	5.5	5.5	5.0	7.5	6.0	6.0	5.5	4.5
	3	6.5	6.5	5.5	7.0	6.5	6.0	6.0	5.0
5 ppm	1	60.5	63.0	64.5	67.5	66.5	62.5	62.5	59.5
	2	60.5	62.0	64.5	66.5	68.0	63.5	64.0	59.0
	3	61.5	62.0	65.0	66.0	69.5	64.5	61.5	58.0
10 ppm	1	65.0	68.5	68.0	71.5	70.0	68.0	67.5	66.0
	2	66.0	66.5	66.0	72.5	71.0	68.0	66.5	65.0
	3	65.5	68.5	69.0	72.5	70.5	67.5	66.0	64.5
15 ppm	1	78.5	81.5	82.5	85.5	83.5	79.5	81.0	79.5
	2	78.0	81.0	82.0	85.5	84.0	78.5	80.0	81.0
	3	77.5	81.5	82.0	85.0	81.5	78.0	80.5	79.0
20 ppm	1	92.5	92.0	94.0	98.0	95.0	93.5	93.5	92.0
	2	85.0	92.0	93.0	96.5	95.0	94.5	89.5	91.5
	3	89.0	91.0	95.0	96.5	94.0	91.5	90.0	91.0

2.2 Copper Concentration (second run)

Sample		Concentration of Copper (mg/kg)							
		Acc 1 (24h)	Acc 2 (48h)	Acc 3 (72h)	Acc 4 (96h)	Dep 1 (120h)	Dep 2 (144h)	Dep 3 (168h)	Dep 4 (192h)
Control	1	12.0	16.0	20.0	23.0	21.5	19.0	15.5	16.0
	2	11.5	17.0	20.5	16.5	20.0	18.5	16.5	16.0
	3	11.5	16.5	21.5	17.5	21.0	17.5	16.5	17.0
5 ppm	1	69.5	72.0	74.5	76.5	76.5	70.0	71.0	70.0
	2	69.0	71.5	74.5	77.5	75.0	71.5	71.0	69.0
	3	68.5	70.5	75.0	76.0	75.0	70.5	70.5	68.5
10 ppm	1	82.5	87.5	88.0	90.0	87.0	85.5	82.0	80.0
	2	83.0	87.0	87.0	89.5	87.0	85.0	81.5	77.5
	3	82.5	88.0	87.5	89.5	87.0	84.5	80.0	78.5
15 ppm	1	80.0	81.5	88.0	89.5	88.5	85.0	82.5	84.5
	2	81.5	82.5	88.5	88.5	87.0	83.5	81.5	82.5
	3	82.5	82.5	88.5	89.0	89.5	82.5	82.0	82.5
20 ppm	1	90.0	94.5	97.0	98.0	92.5	91.5	93.0	91.0
	2	91.5	94.0	96.5	99.0	92.0	91.5	92.0	90.0
	3	91.0	95.0	97.5	99.0	92.0	94.0	90.0	89.5

2.3 Zinc Concentration (first run)

Sample		Concentration of Zinc (mg/kg)							
		Acc 1 (24h)	Acc 2 (48h)	Acc 3 (72h)	Acc 4 (96h)	Dep 1 (120h)	Dep 2 (144h)	Dep 3 (168h)	Dep 4 (192h)
Control	1	40.0	43.0	47.5	42.5	42.5	45.5	42.0	46.0
	2	40.0	41.0	41.5	42.0	35.0	41.5	43.5	42.0
	3	40.5	40.0	36.5	42.5	44.0	41.5	40.0	44.0
10 ppm	1	155.0	169.0	182.0	201.0	200.0	191.5	191.0	189.5
	2	155.5	169.0	186.0	201.0	200.5	191.0	191.0	191.0
	3	154.0	168.0	182.5	200.0	201.5	192.0	190.5	189.5
15 ppm	1	175.0	185.5	201.5	222.0	216.0	215.0	217.5	216.0
	2	175.0	184.0	201.0	221.0	216.0	215.0	217.0	217.0
	3	174.5	184.0	202.0	221.5	216.5	216.0	218.0	216.0
20 ppm	1	194.5	202.0	231.0	240.0	239.5	230.0	236.5	238.0
	2	193.5	201.0	231.0	241.5	239.5	231.5	237.5	238.0
	3	193.5	200.5	230.5	238.0	240.0	231.5	235.0	239.0
25 ppm	1	210.5	218.5	215.5	297.0	294.0	288.0	283.5	284.5
	2	210.0	218.5	219.0	297.0	293.0	288.0	283.5	284.5
	3	211.5	216.5	217.5	296.0	291.5	287.5	283.5	283.5

2.4 Zinc Concentration (second run)

Sample		Concentration of Zinc (mg/kg)							
		Acc 1 (24h)	Acc 2 (48h)	Acc 3 (72h)	Acc 4 (96h)	Dep 1 (120h)	Dep 2 (144h)	Dep 3 (168h)	Dep 4 (192h)
Control	1	38.5	38.0	41.5	41.0	39.5	37.0	42.0	43.5
	2	38.5	38.5	41.0	41.0	38.5	36.5	43.0	44.0
	3	39.0	37.5	41.0	41.0	40.0	37.5	43.0	44.0
10 ppm	1	131.5	137.5	129.0	144.0	142.0	138.0	135.5	131.5
	2	132.5	137.5	130.0	144.0	142.5	139.0	130.0	132.0
	3	132.0	136.5	129.5	143.5	143.5	139.5	128.0	132.0
15 ppm	1	156.5	162.5	179.0	194.0	192.0	188.0	185.5	181.5
	2	156.5	161.5	178.0	193.0	192.0	188.5	185.5	182.5
	3	156.5	160.5	180.0	193.0	192.5	188.5	186.0	182.0
20 ppm	1	178.0	186.0	194.5	204.0	201.0	197.0	193.0	189.5
	2	179.0	187.0	191.5	200.0	201.0	196.0	193.0	188.5
	3	178.5	180.0	191.0	201.5	201.5	196.0	193.0	187.5
25 ppm	1	197.0	206.0	216.5	224.0	218.5	211.5	209.5	202.0
	2	196.5	206.0	215.0	224.5	219.5	210.0	208.0	202.5
	3	195.0	206.5	215.5	223.5	225.0	210.0	208.0	203.0

2.5 Lead Concentration (first run)

Sample		Concentration of Lead (mg/kg)							
		Acc 1 (24h)	Acc 2 (48h)	Acc 3 (72h)	Acc 4 (96h)	Dep 1 (120h)	Dep 2 (144h)	Dep 3 (168h)	Dep 4 (192h)
Control	1	4.5	5.0	4.0	5.5	4.0	4.5	6.0	3.5
	2	4.5	5.5	4.5	6.0	2.5	3.5	3.5	3.5
	3	4.0	6.0	3.5	5.5	3.5	4.0	4.0	4.0
1 ppm	1	11.0	12.0	13.5	17.0	16.5	16.0	15.0	13.5
	2	10.0	11.5	13.0	16.5	16.0	15.5	14.0	12.5
	3	11.5	11.5	13.0	15.5	16.0	16.5	13.0	12.5
2 ppm	1	23.0	24.5	21.5	27.5	25.0	20.0	24.0	24.5
	2	23.5	24.0	25.0	27.0	24.5	19.0	24.0	23.5
	3	22.0	24.0	24.5	27.5	23.0	18.5	23.5	21.5
3 ppm	1	31.5	34.0	39.5	42.0	40.5	37.5	32.0	30.5
	2	31.0	32.5	35.0	41.0	40.0	37.5	32.0	27.5
	3	30.0	33.0	35.0	43.0	40.5	37.5	33.0	28.5
4 ppm	1	44.5	49.0	71.0	87.0	76.5	62.0	53.5	46.5
	2	45.0	45.5	65.0	88.5	76.5	61.5	54.0	49.5
	3	45.0	45.0	65.0	87.5	76.0	61.5	56.0	46.0

2.6 Lead Concentration (second run)

Sample		Concentration of Lead (mg/kg)							
		Acc 1 (24h)	Acc 2 (48h)	Acc 3 (72h)	Acc 4 (96h)	Dep 1 (120h)	Dep 2 (144h)	Dep 3 (168h)	Dep 4 (192h)
Control	1	1.5	1.0	2.0	1.5	2.0	1.5	2.5	2.0
	2	1.5	1.0	1.5	1.0	1.0	1.5	1.5	1.5
	3	2.0	1.0	1.0	1.0	2.0	1.5	2.0	1.0
1 ppm	1	7.0	8.0	11.5	13.0	12.5	11.5	9.5	7.5
	2	6.5	7.5	11.5	12.5	12.5	10.5	9.0	7.5
	3	6.0	7.0	11.5	14.0	12.0	12.0	9.0	7.0
2 ppm	1	19.0	21.5	24.5	27.0	26.0	23.0	20.0	17.5
	2	19.0	21.0	25.0	26.5	25.5	23.0	21.0	16.5
	3	18.5	21.5	25.5	26.0	25.5	22.5	21.5	17.5
3 ppm	1	29.5	33.5	37.0	44.0	39.0	37.0	34.5	31.5
	2	30.5	32.5	37.0	43.0	40.0	37.0	34.0	32.0
	3	30.0	33.0	37.0	43.5	38.0	36.5	33.0	32.5
4 ppm	1	42.0	39.5	53.5	66.0	64.0	61.5	57.0	52.5
	2	42.0	40.0	51.5	66.5	64.0	61.0	56.5	53.0
	3	42.5	40.0	52.0	67.0	63.5	60.0	58.0	53.0

Appendix A-3

Table 3: Mean Concentration of Heavy Metals

3.1 Copper Concentration (first run)

Sample	N	Concentration of Copper (mg/kg)							
		Acc 1 (24 h)	Acc 2 (48h)	Acc 3 (72h)	Acc 4 (96h)	Dep 1 (120h)	Dep 2 (144h)	Dep 3 (168h)	Dep 4 (192h)
Control	3	6.00 ± 0.500	6.17 ± 0.577	5.17 ± 0.289	7.33 ± 0.289	6.17 ± 0.289	5.50 ± 0.866	5.67 ± 0.289	5.33 ± 1.041
5 ppm	3	60.83 ± 0.577	62.33 ± 0.577	64.67 ± 0.289	66.67 ± 0.764	68.00 ± 1.500	63.50 ± 1.000	62.67 ± 1.258	58.83 ± 0.764
10 ppm	3	65.50 ± 0.500	67.83 ± 1.155	67.67 ± 1.528	72.17 ± 0.577	70.50 ± 0.500	67.83 ± 0.289	66.67 ± 0.764	65.17 ± 0.764
15 ppm	3	78.00 ± 0.500	81.33 ± 0.289	82.17 ± 0.289	85.33 ± 0.289	83.00 ± 1.323	78.67 ± 0.764	80.50 ± 0.500	79.83 ± 1.041
20 ppm	3	88.83 ± 3.753	91.67 ± 0.577	94.00 ± 1.000	97.00 ± 0.866	94.67 ± 0.577	93.17 ± 1.528	91.00 ± 2.179	91.50 ± 0.500

3.2 Copper Concentration (second run)

Sample	N	Concentration of Copper (mg/kg)							
		Acc 1 (24 h)	Acc 2 (48h)	Acc 3 (72h)	Acc 4 (96h)	Dep 1 (120h)	Dep 2 (144h)	Dep 3 (168h)	Dep 4 (192h)
Control	3	11.67 ± 0.289	16.50 ± 0.500	20.67 ± 0.764	19.00 ± 3.500	20.83 ± 0.764	18.33 ± 0.764	16.17 ± 0.577	16.33 ± 0.577
5 ppm	3	69.00 ± 0.500	71.33 ± 0.764	74.67 ± 0.289	76.67 ± 0.764	75.50 ± 0.866	70.67 ± 0.764	70.83 ± 0.289	69.17 ± 0.764
10 ppm	3	82.67 ± 0.289	87.50 ± 0.500	87.50 ± 0.500	89.67 ± 0.289	87.00 ± 0.000	85.00 ± 0.500	81.17 ± 1.041	78.67 ± 1.258
15 ppm	3	81.33 ± 1.258	82.17 ± 0.577	88.33 ± 0.289	89.00 ± 0.500	88.33 ± 1.258	83.67 ± 1.258	82.00 ± 0.500	83.17 ± 1.155
20 ppm	3	90.83 ± 0.764	94.50 ± 0.500	97.00 ± 0.500	98.67 ± 0.577	92.17 ± 0.289	92.33 ± 1.443	91.67 ± 1.528	90.17 ± 0.764

3.3 Zinc Concentration (first run)

Sample	N	Concentration of Zinc (mg/kg)							
		Acc 1 (24 h)	Acc 2 (48h)	Acc 3 (72h)	Acc 4 (96h)	Dep 1 (120h)	Dep 2 (144h)	Dep 3 (168h)	Dep 4 (192h)
Control	3	40.17 ± 0.289	41.33 ± 1.528	41.83 ± 5.508	42.33 ± 0.289	40.50 ± 4.822	42.83 ± 2.309	41.83 ± 1.756	44.00 ± 2.000
10 ppm	3	154.83 ± 0.764	168.67 ± 0.577	183.50 ± 2.179	200.67 ± 0.577	200.67 ± 0.764	191.50 ± 0.500	190.83 ± 0.289	190.00 ± 0.866
15 ppm	3	174.83 ± 0.289	184.50 ± 0.866	201.50 ± 0.500	221.50 ± 0.500	216.17 ± 0.289	215.33 ± 0.577	217.50 ± 0.500	216.33 ± 0.577
20 ppm	3	193.83 ± 0.577	201.17 ± 0.764	230.83 ± 0.289	239.83 ± 1.756	239.67 ± 0.289	231.00 ± 0.866	236.33 ± 1.258	238.33 ± 0.577
25 ppm	3	210.67 ± 0.764	217.83 ± 1.155	217.33 ± 1.756	296.67 ± 0.577	292.83 ± 1.258	287.83 ± 0.289	283.50 ± 0.000	284.17 ± 0.577

3.4 Zinc Concentration (second run)

Sample	N	Concentration of Zinc (mg/kg)							
		Acc 1 (24 h)	Acc 2 (48h)	Acc 3 (72h)	Acc 4 (96h)	Dep 1 (120h)	Dep 2 (144h)	Dep 3 (168h)	Dep 4 (192h)
Control	3	38.67 ± 0.289	38.00 ± 0.500	41.17 ± 0.289	41.00 ± 0.000	39.33 ± 0.764	37.00 ± 0.500	42.67 ± 0.577	43.83 ± 0.289
10 ppm	3	132.00 ± 0.500	137.17 ± 0.577	129.50 ± 0.500	143.83 ± 0.289	142.67 ± 0.764	138.83 ± 0.764	131.17 ± 3.884	131.83 ± 0.289
15 ppm	3	156.50 ± 0.000	161.50 ± 0.577	179.00 ± 1.000	193.33 ± 0.577	192.17 ± 0.289	188.33 ± 0.289	185.67 ± 0.289	182.00 ± 0.500
20 ppm	3	178.50 ± 0.500	184.33 ± 3.786	192.33 ± 1.893	201.83 ± 2.021	201.17 ± 0.289	196.33 ± 0.577	193.00 ± 0.000	188.50 ± 1.000
25 ppm	3	196.17 ± 1.041	206.17 ± 0.289	215.67 ± 0.764	224.00 ± 0.500	221.00 ± 3.500	210.50 ± 0.866	208.50 ± 0.866	202.50 ± 0.500

3.5 Lead Concentration (first run)

Sample	N	Concentration of Lead (mg/kg)							
		Acc 1 (24 h)	Acc 2 (48h)	Acc 3 (72h)	Acc 4 (96h)	Dep 1 (120h)	Dep 2 (144h)	Dep 3 (168h)	Dep 4 (192h)
Control	3	4.33 ± 0.289	5.50 ± 0.500	4.00 ± 0.500	5.67 ± 0.289	3.33 ± 0.764	4.00 ± 0.500	4.50 ± 1.323	3.67 ± 0.289
1 ppm	3	10.83 ± 0.764	11.67 ± 0.289	13.17 ± 0.289	16.33 ± 0.764	16.17 ± 0.289	16.00 ± 0.500	14.00 ± 1.000	12.83 ± 0.577
2 ppm	3	22.83 ± 0.764	24.17 ± 0.289	23.67 ± 1.893	27.33 ± 0.289	24.17 ± 1.041	19.17 ± 0.764	23.83 ± 0.289	23.17 ± 1.528
3 ppm	3	30.83 ± 0.764	33.17 ± 0.764	36.50 ± 2.598	42.00 ± 1.000	40.33 ± 0.289	37.50 ± 0.000	32.33 ± 0.577	28.83 ± 1.528
4 ppm	3	44.83 ± 0.289	46.50 ± 2.179	67.00 ± 3.464	87.67 ± 0.764	76.33 ± 0.289	61.67 ± 0.289	54.50 ± 1.323	47.33 ± 1.893

3.6 Lead Concentration (second run)

Sample	N	Concentration of Lead (mg/kg)							
		Acc 1 (24 h)	Acc 2 (48h)	Acc 3 (72h)	Acc 4 (96h)	Dep 1 (120h)	Dep 2 (144h)	Dep 3 (168h)	Dep 4 (192h)
Control	3	1.67 ± 0.289	1.00 ± 0.000	1.50 ± 0.500	1.17 ± 0.289	1.67 ± 0.577	1.50 ± 0.000	2.00 ± 0.500	1.50 ± 0.500
1 ppm	3	6.50 ± 0.500	7.50 ± 0.500	11.50 ± 0.000	13.17 ± 0.764	12.33 ± 0.289	11.33 ± 0.764	9.17 ± 0.289	7.33 ± 0.289
2 ppm	3	18.83 ± 0.289	21.33 ± 0.289	25.00 ± 0.500	26.50 ± 0.500	25.67 ± 0.289	22.83 ± 0.289	20.83 ± 0.764	17.17 ± 0.577
3 ppm	3	30.00 ± 0.500	33.00 ± 0.500	37.00 ± 0.000	43.50 ± 0.500	39.00 ± 1.000	36.83 ± 0.289	33.83 ± 0.764	32.00 ± 0.500
4 ppm	3	42.17 ± 0.289	39.83 ± 0.289	52.33 ± 1.041	66.50 ± 0.500	63.83 ± 0.289	60.83 ± 0.764	57.17 ± 0.764	52.83 ± 0.289

Appendix A-4
Table 4: Mortality

4.1 Copper (first run)

Sample	Motality (number of fish dies)							
	24 hours	48 hours	72 hours	96 hours	120 hours	144 hours	168 hours	192 hours
Control	0	0	1	1	0	0	0	1
5 ppm	0	0	0	1	1	0	2	0
10 ppm	0	2	0	0	0	0	0	0
15 ppm	1	1	1	0	0	0	1	0
20 ppm	0	0	0	2	0	1	0	0

4.2 Copper (second run)

Sample	Motality (number of fish dies)							
	24 hours	48 hours	72 hours	96 hours	120 hours	144 hours	168 hours	192 hours
Control	0	0	0	0	0	0	0	1
5 ppm	0	0	1	0	0	0	0	0
10 ppm	3	0	0	1	0	0	0	0
15 ppm	0	0	0	0	1	1	0	0
20 ppm	0	0	2	0	0	0	1	1

4.3 Zinc (first run)

Sample	Mortality (number of fish dies)							
	24 hours	48 hours	72 hours	96 hours	120 hours	144 hours	168 hours	192 hours
Control	0	0	0	0	0	2	0	1
10 ppm	1	1	0	0	0	1	0	0
15 ppm	0	0	0	3	0	0	1	0
20 ppm	0	0	0	0	2	0	1	1
25ppm	1	1	0	0	0	2	0	0

4.4 Zinc (second run)

Sample	Mortality (number of fish dies)							
	24 hours	48 hours	72 hours	96 hours	120 hours	144 hours	168 hours	192 hours
Control	0	0	0	0	0	0	0	1
10 ppm	0	0	1	0	1	1	0	0
15 ppm	2	0	0	1	0	0	0	0
20 ppm	0	0	0	3	0	1	0	0
25ppm	0	0	2	2	0	0	0	0

4.5 Lead (first run)

Sample	Mortality (number of fish dies)							
	24 hours	48 hours	72 hours	96 hours	120 hours	144 hours	168 hours	192 hours
Control	0	0	0	0	1	0	0	0
1 ppm	0	0	0	0	0	0	0	0
2 ppm	0	0	0	0	1	1	0	0
3 ppm	1	1	0	0	2	0	0	0
4 ppm	1	0	0	2	0	0	0	1

4.6 Lead (second run)

Sample	Mortality (number of fish dies)							
	24 hours	48 hours	72 hours	96 hours	120 hours	144 hours	168 hours	192 hours
Control	0	0	0	0	0	0	0	0
1 ppm	0	0	3	0	0	1	0	0
2 ppm	2	1	0	0	0	0	1	0
3 ppm	1	0	0	2	0	0	0	0
4 ppm	3	1	0	0	0	1	0	0

APPENDIX B
Two-Factor Without Replication (ANOVA)

Appendix B-1

Copper (first run) variation with control sample

Anova: Two-Factor Without Replication

SUMMARY	*Count*	*Sum*	*Average*	*Variance*
Row 1	8	47.5	5.9375	0.888392857
Row 2	8	45.5	5.6875	0.78125
Row 3	8	49	6.125	0.410714286
Row 4	8	506.5	63.3125	7.566964286
Row 5	8	508	63.5	8.857142857
Row 6	8	508	63.5	12.28571429
Row 7	8	544.5	68.0625	4.245535714
Row 8	8	541.5	67.6875	7.138392857
Row 9	8	544	68	7.214285714
Row 10	8	651.5	81.4375	5.459821429
Row 11	8	650	81.25	6.571428571
Row 12	8	645	80.625	5.982142857
Row 13	8	750.5	93.8125	3.924107143
Row 14	8	737	92.125	13.125
Row 15	8	738	92.25	6.857142857
Column 1	15	897.5	59.83333333	881.202381
Column 2	15	928	61.86666667	944.3380952
Column 3	15	941	62.73333333	1007.066667
Column 4	15	985.5	65.7	1031.957143
Column 5	15	967	64.46666667	1008.516667
Column 6	15	926	61.73333333	960.102381
Column 7	15	919.5	61.3	938.85
Column 8	15	902	60.13333333	943.802381

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	107812.7417	14	7700.910119	3611.673101	3.1E-126	1.793981
Columns	430.1979167	7	61.45684524	28.82283151	3.39E-21	2.104448
Error	208.9583333	98	2.132227891			
Total	108451.8979	119				

Appendix B-2

Copper (second run) variation with control sample

Anova: Two-Factor Without Replication

SUMMARY	*Count*	*Sum*	*Average*	*Variance*
Row 1	8	143	17.875	13.19642857
Row 2	8	136.5	17.0625	7.888392857
Row 3	8	139	17.375	9.482142857
Row 4	8	580	72.5	8.571428571
Row 5	8	579	72.375	9.125
Row 6	8	574.5	71.8125	9.28125
Row 7	8	682.5	85.3125	11.99553571
Row 8	8	677.5	84.6875	14.85267857
Row 9	8	677.5	84.6875	16.06696429
Row 10	8	679.5	84.9375	12.17410714
Row 11	8	675.5	84.4375	9.316964286
Row 12	8	679	84.875	11.76785714
Row 13	8	747.5	93.4375	8.174107143
Row 14	8	746.5	93.3125	9.138392857
Row 15	8	748	93.5	12.21428571
Column 1	15	1006.5	67.1	875.65
Column 2	15	1056	70.4	839.7571429
Column 3	15	1104.5	73.63333333	806.0880952
Column 4	15	1119	74.6	882.4714286
Column 5	15	1091.5	72.76666667	755.9595238
Column 6	15	1050	70	767.9642857
Column 7	15	1025.5	68.36666667	777.052381
Column 8	15	1012.5	67.5	751.6428571

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	90106.27917	14	6436.162798	2206.009079	9.1155E-116	1.79398138
Columns	856.7979167	7	122.3997024	41.95276956	8.98549E-27	2.104448182
Error	285.9208333	98	2.917559524			
Total	91248.99792	119				

Appendix B-3

Zinc (first run) variation with control sample

Anova: Two-Factor Without Replication

SUMMARY	*Count*	*Sum*	*Average*	*Variance*
Row 1	8	349	43.625	6.125
Row 2	8	326.5	40.8125	6.495535714
Row 3	8	329	41.125	6.125
Row 4	8	1479	184.875	249.1964286
Row 5	8	1485	185.625	246.9107143
Row 6	8	1478	184.75	264.6428571
Row 7	8	1648.5	206.0625	295.8169643
Row 8	8	1646	205.75	302.5
Row 9	8	1648.5	206.0625	312.3883929
Row 10	8	1811.5	226.4375	320.03125
Row 11	8	1813.5	226.6875	347.3526786
Row 12	8	1808	226	335.2857143
Row 13	8	2091.5	261.4375	1513.959821
Row 14	8	2093.5	261.6875	1467.852679
Row 15	8	2087.5	260.9375	1456.102679
Column 1	15	2323	154.8666667	3897.409524
Column 2	15	2440.5	162.7	4235.064286
Column 3	15	2625	175	5023.5
Column 4	15	3003	200.2	7768.957143
Column 5	15	2969.5	197.9666667	7690.695238
Column 6	15	2905.5	193.7	7176.528571
Column 7	15	2910	194	7183.428571
Column 8	15	2918.5	194.5666667	7095.530952

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	683278.625	14	48805.61607	269.9642786	1.34255E-71	1.79398138
Columns	32198.525	7	4599.789286	25.4433587	1.7197E-19	2.104448182
Error	17716.975	98	180.7854592			
Total	733194.125	119				

Appendix B-4

Zinc (second run) variation with control sample

Anova: Two-Factor Without Replication

SUMMARY	*Count*	*Sum*	*Average*	*Variance*
Row 1	8	321	40.125	4.982142857
Row 2	8	321	40.125	6.553571429
Row 3	8	323	40.375	5.625
Row 4	8	1089	136.125	27.98214286
Row 5	8	1087.5	135.9375	31.10267857
Row 6	8	1084.5	135.5625	37.38839286
Row 7	8	1439	179.875	185.125
Row 8	8	1437.5	179.6875	188.4955357
Row 9	8	1439	179.875	195.6964286
Row 10	8	1543	192.875	69.91071429
Row 11	8	1536	192	52.64285714
Row 12	8	1529	191.125	76.98214286
Row 13	8	1685	210.625	79.83928571
Row 14	8	1682	210.25	83.35714286
Row 15	8	1686.5	210.8125	103.2098214
Column 1	15	2105.5	140.3666667	3266.159524
Column 2	15	2181.5	145.4333333	3660.17381
Column 3	15	2273	151.5333333	4114.730952
Column 4	15	2412	160.8	4581.314286
Column 5	15	2389	159.2666667	4568.066667
Column 6	15	2313	154.2	4303.564286
Column 7	15	2283	152.2	3943.957143
Column 8	15	2246	149.7333333	3613.852381

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	445455.842	14	31818.2744	953.684564	5.09E-98	1.79398138
Columns	4772.625	7	681.8035714	20.4356004	1.09E-16	2.104448182
Error	3269.625	98	33.36352041			
Total	453498.092	119				

Appendix B-5

Lead (first run) variation with control sample

Anova: Two-Factor Without Replication

SUMMARY	*Count*	*Sum*	*Average*	*Variance*
Row 1	8	37	4.625	0.696428571
Row 2	8	33.5	4.1875	1.352678571
Row 3	8	34.5	4.3125	0.852678571
Row 4	8	114.5	14.3125	4.709821429
Row 5	8	109	13.625	5.267857143
Row 6	8	109.5	13.6875	4.066964286
Row 7	8	190	23.75	5.214285714
Row 8	8	190.5	23.8125	5.066964286
Row 9	8	184.5	23.0625	6.745535714
Row 10	8	287.5	35.9375	20.17410714
Row 11	8	276.5	34.5625	21.88839286
Row 12	8	280.5	35.0625	25.10267857
Row 13	8	490	61.25	242.7857143
Row 14	8	485.5	60.6875	241.3526786
Row 15	8	482	60.25	243
Column 1	15	341	22.73333333	221.8880952
Column 2	15	363	24.2	233.2071429
Column 3	15	433	28.86666667	518.4452381
Column 4	15	537	35.8	876.1714286
Column 5	15	481	32.06666667	679.2809524
Column 6	15	415	27.66666667	433.2738095
Column 7	15	387.5	25.83333333	314.2380952
Column 8	15	347.5	23.16666667	237.6309524

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	45601.8542	14	3257.2753	88.7677727	3.96858E-49	1.79398138
Columns	2201.89167	7	314.55595	8.572327707	3.6429E-08	2.104448182
Error	3596.04583	98	36.694345			
Total	51399.7917	119				

Appendix B-6

Lead (second run) variation with control sample

Anova: Two-Factor Without Replication

SUMMARY	*Count*	*Sum*	*Average*	*Variance*
Row 1	8	14	1.75	0.214285714
Row 2	8	10.5	1.3125	0.066964286
Row 3	8	11.5	1.4375	0.245535714
Row 4	8	80.5	10.0625	5.602678571
Row 5	8	77.5	9.6875	5.709821429
Row 6	8	78.5	9.8125	8.709821429
Row 7	8	178.5	22.3125	11.56696429
Row 8	8	177.5	22.1875	11.92410714
Row 9	8	178.5	22.3125	10.42410714
Row 10	8	286	35.75	20.78571429
Row 11	8	286	35.75	18.42857143
Row 12	8	283.5	35.4375	17.88839286
Row 13	8	436	54.5	94.85714286
Row 14	8	434.5	54.3125	93.99553571
Row 15	8	436	54.5	91.78571429
Column 1	15	297.5	19.83333333	238.1666667
Column 2	15	308	20.53333333	231.5166667
Column 3	15	382	25.46666667	348.3380952
Column 4	15	452.5	30.16666667	566.202381
Column 5	15	427.5	28.5	503.3928571
Column 6	15	400	26.66666667	461.7380952
Column 7	15	369	24.6	409.3642857
Column 8	15	332.5	22.16666667	366.4166667

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	42450.05417	14	3032.146726	228.2531246	3.88777E-68	1.79398138
Columns	1443.591667	7	206.227381	15.52432924	1.53148E-13	2.104448182
Error	1301.845833	98	13.28414116			
Total	45195.49167	119				